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Analyses of Sporads Types at Different Bud Development Stages of *Phalaenopsis* Orchids

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This study aimed to investigate sporad types of pollinia collected from flower buds at different development stages to determine the optimal timing for unreduced male gamete induction. Pollinia collected at different flower development stages in 6 *Phalaenopsis* species, including *P. amabilis, P. amboinensis* var. *flava, P. equestris, P. hieroglyphica, P. venosa* and *P. violacea*, were used to examine the distribution of sporad types. The results showed that the highest percentages of monad were observed when the sizes of flower buds were about or less than 5 mm in *P. amabilis, P. equestris, P. hieroglyphica* and *P. venosa*, and about or less than 7.5 mm in *P. amboinensis* var. *flava* and *P. violacea*. Accordingly, those sized flower buds might be suitable for subsequent unreduced male gamete induction needed for developing an efficient sexual polyploidization breeding scheme in *Phalaenopsis*.

Key words: flower bud, monad, pollinium

INTRODUCTION

Phalaenopsis orchid has been a favorite consumer product due to its attractive color, graceful and long–lasting flowers. It is commonly recognized that *Phalaenopsis* orchid is economically the most important mass–marketed potted–plant orchid. Polyploid cultivars of *Phalaenopsis* have superior horticultural traits such as larger size of the flowers and better shape. The *Phalaenopsis* cultivars used for breeding are usually divided into two groups including the standard big flower group such as derivatives of *P. amabilis* and *P. schilleriana*, the novelty group such as descendants of *P. violacea, P. amboinensis* and *P. venosa* (Tang and Chen, 2007).

Polyploid has played an important role in the improvement of many plant species and hybrids. The field of floriculture has probably benefited the most because first one polyploidy can increase genetic variability, and second polyploidy tends to increase the size and substance and improve the form of flowers. Because of these characteristics, breeders have unconsciously selected and used polyploid forms as parents (Grlesbach, 1985).

Sexual polyploidy in plant breeding, $2n$ gametes (including $2n$ pollen and $2n$ eggs) play an important role (Ramanna and Jacobsen, 2003), can overcome the barriers to hybridization, or enable future generations to restore. Suitable flower bud before treatment flower bud with antimicrotubule agent reported in many crops such as lily, begonia and tulip (Akutsu *et al*., 2007; Barba–Gonzalez *et al*., 2006; Dewitte *et al*., 2010). Akutsu *et al*. (2007) showed that effects were optimal when treatments started during pollen mother cell progression to metaphase I. In tulip microsporogenesis occurs inside the bulb, in genera where microsporogenesis starts within visible flower buds, antimicrotubule agent starts within visible flower buds (Dewitte *et al*., 2010).

This study aimed to determine which flower bud length of *Phalaenopsis* that suitable for use to induction of male unreduced gamete production for developing an efficient sexual polyploidization breeding scheme in *Phalaenopsis*.

MATERIALS AND METHODS

Plant materials

Six *Phalaenopsis* species (2n=2x=38), including *P. amabilis, P. amboinensis* var. *flava, P. equestris, P. hieroglyphica, P. venosa* and *P. violacea*, were used in this experiment (Fig. 1). Plants were cultivated in 10 cm diameter pots in a greenhouse at the Horticultural Technology Center, National Chiayi University, Chiayi City, Taiwan (R.O.C.).

Observation of sporad types at different flower bud development stages in *Phalaenopsis* orchids

To quantify the number of different types of sporads in *Phalaenopsis*, pollinia were stained with lactophenol–acid fuchsin solution (Lim *et al*., 2001). Pollinia from different sized flower buds were fixed with 95% ethanol–glacial acetic acid (3:1 v/v) solution for 24 hours, hydrolyzed in 1 N HCl for 20 minutes at 60°C, rinsed three times with distilled water, and stained with lactophenol–acid fuchsin solution containing 50 mg acid fuchsin, 5 g phenol, 5 mL lactic acid, 25 mL glycerol, 10 mL 95% ethanol, and 60 mL distilled water (Lim *et al*., 2001). Materials for observation were examined under microscope (Axio version Rel.4.5,

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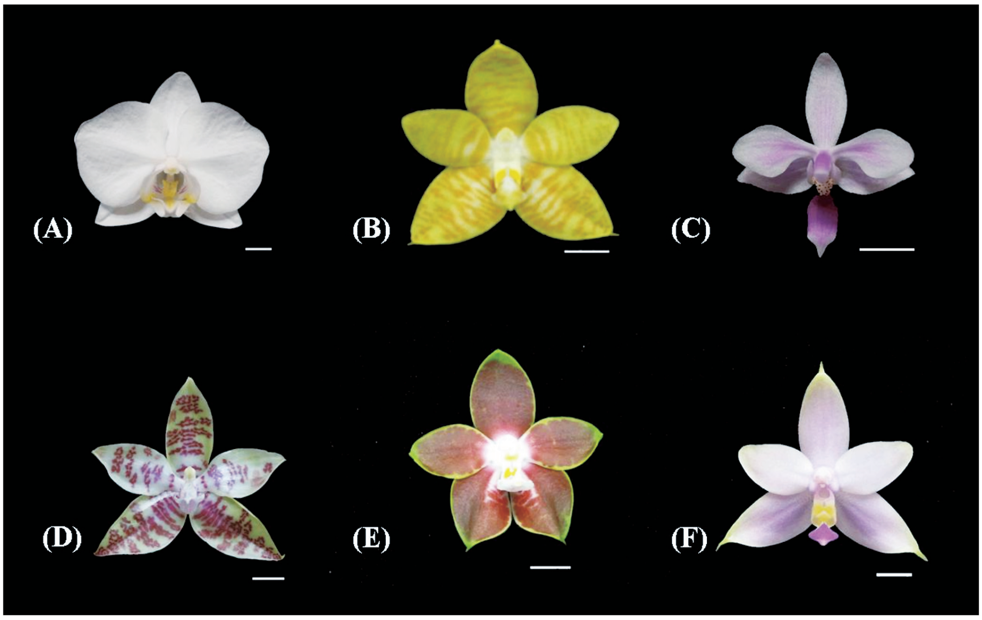

Fig. 1. Flower of plant materials: (A) *Phalaenopsis amabilis*; (B) *P. amboinensis* var. *flava*; (C) P. equestris; (D) *P. hieroglyphica*; (E) *P. venosa* and (F) *P. violacea*. Bar=1 cm.

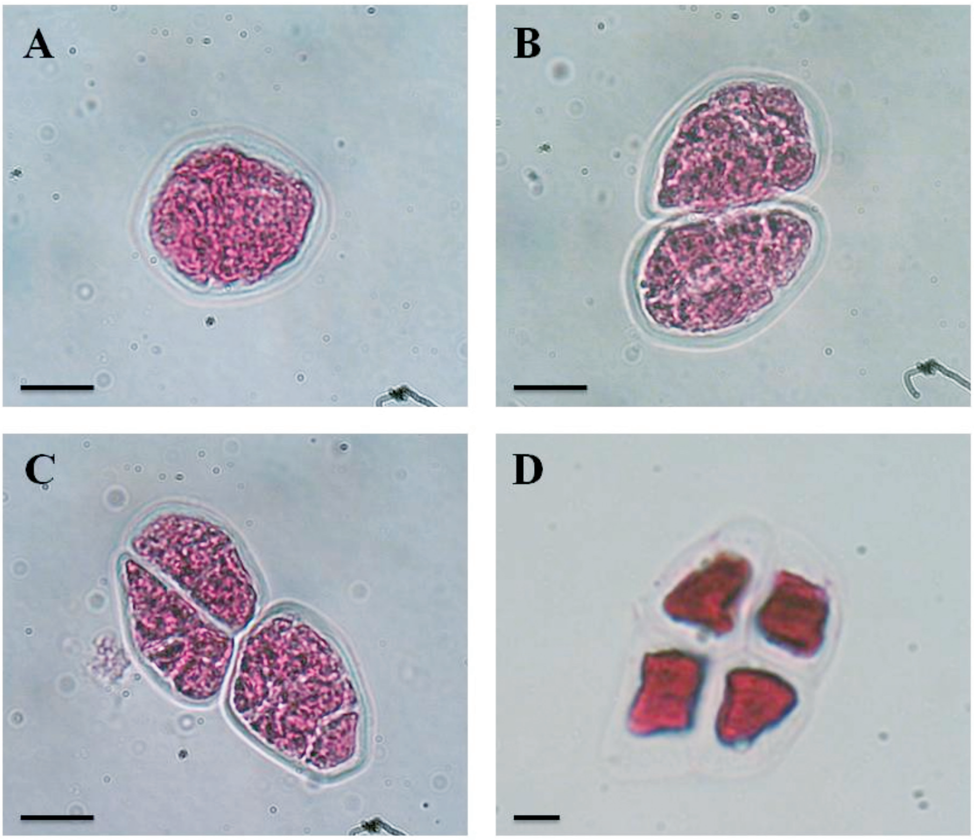

Fig. 2. Lactophenol–acid fuchsin staining of sporads in *Phalaenopsis*. (A) Monad, (B) Dyad, (C) Triad, (D) Tetrad. Bar=5 μm.

Germany) with a CCD camera (Cannon A620, Japan). One thousand sporads were categorized according to the sporad types, and at least three different plants were used for examination. Pictures were also taken using a digital camera (Cannon A620, Japan). Sporad types were classified as monad, dyad, triad, tetrad according to the number of nuclei presented (1 to 4 nuclei) respectively (Fig. 2).

RESULTS

The sporad types of pollinia collected from flower buds at different development stages of 6 species *Phalaenopsis* were observed. Samples of pollinia from different flower bud size of *P. amabilis, P. equestris, P. hieroglyphica, P. amboinensis* var. *flava, P. venosa* and *P. violacea* were stained with lactophenol–acid fuchsin to quantify the different sporad types at least for one thousand sporads. Sporad types were classified as monad, dyad, triad, tetrad according to the number of nuclei presented within the structure (Fig. 2).

Sporad types of *P. amabilis*

Results from sporad observations in *P. amabilis* showed that sporad types of pollinia divided into 75.2% of monad and 24.8% of dyad at flower bud size of 5.0±0.5 mm, 10.5% of monad, 87.8% of dyad and 1.7% of tetrad at flower bud size of 7.5±0.5 mm, less than 2% of dyad and 98.2–98.8% of tetrad at flower bud sizes of 10.0±0.5 mm and 12.5±0.5 mm, and 100% of tetrad at flower bud sizes bigger than 15.0±0.5 mm to flower fully open (Table 1, Fig. 3A).

Sporad types of *P. amboinensis* var. *flava*

Results from sporad observations in *P. amboinensis* var. *flava* showed that sporad types of pollinia divided into 98.5% of monad and 1.5% of dyad at flower bud size of 7.5±0.5 mm, 39.3% of monad, 58.8% of dyad, 0.3% of triad and 1.6% of tetrad at flower bud size of 10.0±0.5 mm, 31.5% of monad, 59.3% of dyad, 0.6% of triad and 8.6% of tetrad at flower bud size of 12.5±0.5 mm, 6.1% of monad, 35.2% of dyad, 0.5% of triad and 58.2% of tetrad at flower bud size of 15.0±0.5 mm, In addition, flower bud size bigger than 20.0±0.5 mm to flower fully open showed tetrad reach to 99.3–99.6% and triad 0.5–0.7% (Table 2, Fig. 3B).

Sporad types of *P. equestris*

Results from sporad observations in *P. equestris* showed that sporad types of pollinia divided into 90.7%

Table 1. Distribution of sporad types of pollinia collected from flower buds at development stages in *Phalaenopsis amabilis*

Flower bud length (mm)	Total number of sporads observed	Number (percentage) of the various sporad types							
		Monad		Dyad		Triad		Tetrad	
5.0±0.5	1213	912	(75.2)	301	(24.8)	0	(0)	0	(0)
7.5±0.5	1273	135	(10.5)	1117	(87.8)	0	(0)	21	(1.7)
10.0±0.5	1100	0	(0)	20	(1.8)	0	(0)	1080	(98.2)
12.5±0.5	1260	0	(0)	15	(1.2)	0	(0)	1245	(98.8)
15.0±0.5	1080	0	(0)	0	(0)	0	(0)	1080	(100)
17.5±0.5	1016	0	(0)	0	(0)	0	(0)	1016	(100)
Blooming	1012	0	(0)	0	(0)	0	(0)	1012	(100)

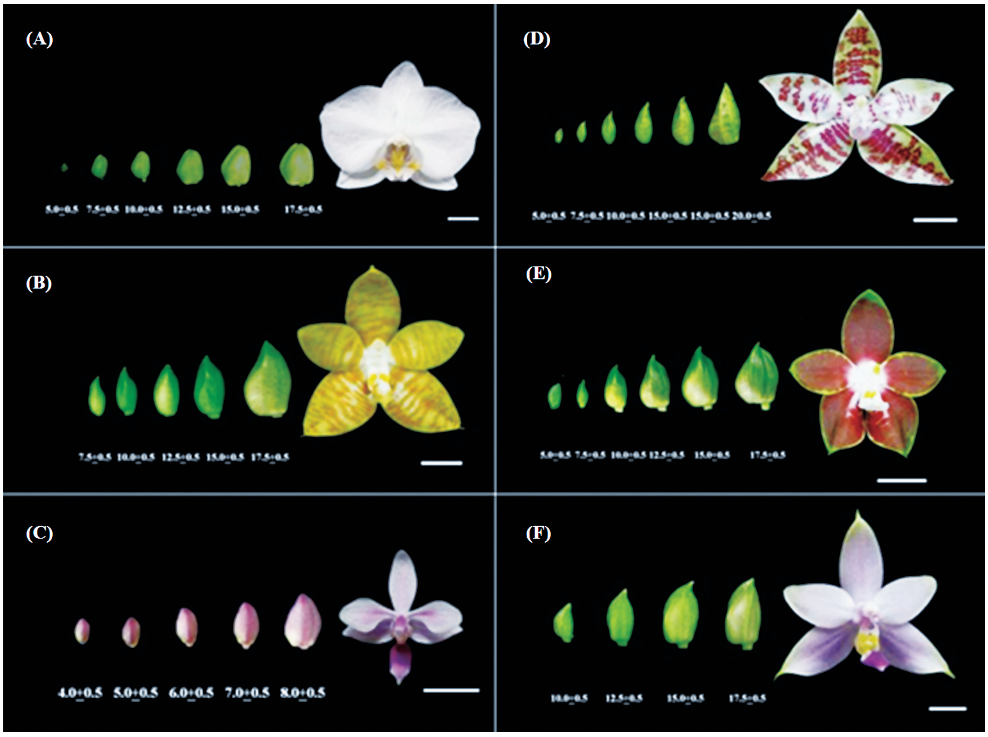

Fig. 3. Flower buds at different development stages of 6 species *Phalaenopsis*
(A) *P. amabilis*; (B) *P. amboinensis* var. *flava*; (C) *P. equestris*; (D) *P. hieroglyphica*; (E) *P. venosa* and (F) *P. violacea*. Bar=1 cm.

Table 2. Distribution of sporad types of pollinia collected from flower buds at development stages in *Phalaenopsis amboinensis* var. *flava*

Flower bud length (mm)	Total number of sporads observed	Number (percentage) of the various sporad types							
		Monad		Dyad		Triad		Tetrad	
7.5±0.5	1033	1017	(98.5)	16	(1.5)	0	(0)	0	(0)
10.0±0.5	1002	394	(39.3)	589	(58.8)	3	(0.3)	16	(1.6)
12.5±0.5	1081	340	(31.5)	642	(59.3)	6	(0.6)	93	(8.6)
15.0±0.5	1018	62	(6.1)	38	(35.2)	6	(0.5)	592	(58.2)
20.0±0.5	1084	0	(0)	0	(0)	7	(0.5)	1077	(99.6)
Blooming	1066	0	(0)	0	(0)	7	(0.7)	1059	(99.3)

Table 3. Distribution of sporad types of pollinia collected from flower buds at development stages in *Phalaenopsis equestris*

Flower bud length (mm)	Total number of sporads observed	Number (percentage) of the various sporad types							
		Monad		Dyad		Triad		Tetrad	
4.0±0.5	1035	939	(90.7)	96	(9.3)	0	(0)	0	(0)
5.0±0.5	1320	963	(73.0)	357	(27.0)	0	(0)	0	(0)
6.0±0.5	1016	80	(7.9)	928	(91.3)	8	(0.8)	0	(0)
7.0±0.5	1640	0	(0)	464	(28.3)	64	(3.9)	1112	(67.8)
8.0±0.5	1112	0	(0)	8	(0.7)	72	(6.5)	1032	(92.8)
Blooming	1010	0	(0)	0	(0.0)	50	(5.0)	960	(95.0)

Table 4. Distribution of sporad types of pollinia collected from flower buds at development stages in *Phalaenopsis hieroglyphica*

Flower bud length (mm)	Total number of sporads observed	Number (percentage) of the various sporad types							
		Monad		Dyad		Triad		Tetrad	
5.0±0.5	1179	1056	(89.6)	123	(10.4)	0	(0)	0	(0)
7.5±0.5	1072	581	(54.2)	469	(43.8)	22	(2.0)	0	(0)
10.0±0.5	1149	512	(44.6)	496	(43.2)	18	(1.5)	123	(10.7)
15.0±0.5	1120	180	(16.1)	822	(73.4)	16	(1.4)	102	(9.1)
17.5±0.5	1332	36	(2.7)	100	(7.5)	17	(1.3)	1179	(88.5)
20.0±0.5	1112	0	(0)	0	(0)	15	(1.3)	1097	(98.7)
Blooming	1050	0	(0)	0	(0)	13	(1.2)	1037	(98.8)

Table5. Distribution of sporad types of pollinia collected from flower buds at development stages in *Phalaenopsis venosa*

Flower bud length (mm)	Total number of sporads observed	Number (percentage) of the various sporad types							
		Monad		Dyad		Triad		Tetrad	
5.0±0.5	1186	784	(66.1)	400	(33.7)	0	(0)	2	(0.2)
7.5±0.5	1096	92	(8.4)	463	(42.2)	0	(0)	541	(49.4)
10.0±0.5	1081	43	(4.0)	103	(9.5)	0	(0)	935	(86.5)
12.5±0.5	1024	0	(0)	20	(2.0)	0	(0)	1004	(98.0)
15.0±0.5	1053	0	(0)	13	(1.2)	0	(0)	1040	(98.8)
15.5±0.5	1025	0	(0)	0	(0)	0	(0)	1025	(100)
Blooming	1188	0	(0)	0	(0)	0	(0)	1118	(100)

Table 6. Distribution of sporad types of pollinia collected from flower buds at development stages in *Phalaenopsis violacea*

Flower bud length (mm)	Total number of sporads observed	Number (percentage) of the various sporad types							
		Monad		Dyad		Triad		Tetrad	
7.5±0.5	1141	930	(81.5)	211	(18.5)	0	(0)	0	(0.0)
10.0±0.5	1018	200	(19.6)	818	(80.4)	0	(0)	0	(0.0)
12.5±0.5	1063	0	(0)	120	(11.3)	0	(0)	943	(88.7)
15.0±0.5	1002	0	(0)	0	(0.0)	0	(0)	1002	(100)
Blooming	1105	0	(0)	0	(0.0)	0	(0)	1105	(100)

of monad and 9.3% of dyad at flower bud size of 4.0±0.5 mm, 73.0% of monad and 27.0% of dyad at flower bud size of 5.0±0.5 mm, 7.9% of monad, 91.3% of dyad and 0.8% of triad at flower bud size of 6.0±0.5 mm, 28.3% of dyad, 3.9% of triad and 67.8% of tetrad at flower bud size of 7.0±0.5 mm, 0.7% of dyad, 6.5% of triad and 92.8% of tetrad at flower bud size of 8.0±0.5 mm, In addition, flower fully open showed 5% of triad and 95% of tetrad (Table 3, Fig. 3C).

Sporad types of *P. hieroglyphica*

Results from sporad observations in *P. hieroglyphica* showed that sporad types of pollinia divided into 89.6% of monad and 10.4% of dyad at flower bud size of 5.0±0.5 mm, 54.2% of monad, 43.8% of dyad and 2.0% of triad at flower bud size of 7.5±0.5 mm, 44.6% of monad, 43.2% of dyad, 1.5% of triad and 10.7% of tetrad at flower bud size of 10.0±0.5 mm, 16.1% of monad, 73.4% of dyad, 1.4% of triad and 9.1% of tetrad at flower bud size of 15.0±0.5 mm, 2.7% of monad, 7.5% of dyad, 1.3% of triad and 88.5% of tetrad at flower bud size of 17.5±0.5 mm, 1.3% of triad and 98.7% of tetrad at flower bud size of 20.0±0.5 mm, In addition, flower fully open showed 1.2% of triad and 98.8% of tetrad (Table 4, Fig. 3D).

Sporad types of in *P. venosa*

Results from sporad observations in *P. venosa* showed that sporad types of pollinia divided into 66.1% of monad, 33.7% of dyad and 0.2% of tetrad at flower bud size of 5.0±0.5 mm, 8.4% of monad, 42.2% of dyad and 49.4% of tetrad at flower bud size of 7.5±0.5 mm, 4.0% of monad, 9.5% of dyad and 86.5% of tetrad at flower bud size of 10.0±0.5 mm, 2.0% of dyad and 98.0% of tetrad at flower bud size of 12.5±0.5 mm, 1.2% of dyad and 98.8% of tetrad at flower bud size of 15.0±0.5 mm, 100% of tetrad at flower bud sizes of 15.5±0.5 mm to flower fully open (Table 5, Fig. 3E).

Sporad types of *P. violacea*

Results from sporad observations in *P. violacea* showed that sporad types of pollinia divided into 81.5% of monad and 18.5% of dyad at flower bud size of 7.5±0.5 mm, 19.6% of monad and 80.4% of dyad at flower bud size of 10.0±0.5 mm, 11.3% of dyad and 88.7% of tetrad at flower bud size of 12.5±0.5 mm, 100% of tetrad at flower bud sizes of 15.0±0.5 mm to flower fully open (Table 6, Fig. 3F).

DISCUSSION

This study aimed to evaluate the optimal timing for unreduced male gamete induction based on the observation of sporad types of pollinia collected at different flower bud development stages in *Phalaenopsis* orchids. Using the immature flower buds with suitable sizes treated with chemical (Dewitte *et al.*, 2010; Li *et al.*, 2008; Wu *et al.*, 2007; Xiao *et al.*, 2007; Zhong *et al.*, 2010) or physical methods (Akutsu *et al.*, 2007; Barba–Gonzales *et al.*, 2006; Kitamura *et al.*, 2009; Okazaki *et al.*, 2005; Sato *et al.*, 2010) to facilitate the successful induction of 2n gametes is an important strategy. Akutsu *et al.* (2007) reported lily bud length could be used to judge the microspore mother cell division for unreduced male gamete induction, and a few 2*n* pollen grains were induced by nitrous oxide (N_2O) treatment using plants with anthers in prophase I, whereas mixed pollen grains of differing size were produced using plants undergoing meiotic metaphase predominantly in anthers. In similar idea, we tried to analyze the relationship between bud size and meiotic stage in *Phalaenopsis* orchids. However we just simply observed sporad types of pollinia instead of detailed, labor–consuming and time–costing examination of microspore mother cell progression. In addition, percentage of tetrads also can use as a determination for selecting pollen donor plant in *Phalaenopsis* breeding (Bolaños–Villegas *et al.*, 2008; Chou, 2013; Wongprichachan *et al.*, 2012).

Our results showed that distributions of sporad types of pollinia collected from flower buds at development stages varied among six *Phalaenopsis* species studied in this experiment. The highest percentages of monad were observed when the sizes of flower buds were about or less than 5.0 mm in *P. amabilis, P. equestris, P. hieroglyphica* and *P. venosa*, and about or less than 7.5 mm in *P. amboinensis* var. *flava* and *P. violacea* (Table 1–6). The results were also similar with those of Chou (2013)'s study. We have previously shown that N_2O treatment with flower buds sized about 4.5–5.0 mm is an effective method to induce unreduced gamete (Wongprichachan *et al.*, 2013a) and polyploid (Wongprichachan *et al.*, 2013b) in *P. amabilis*.

Since the distribution of sporad types of pollinia collected from flower buds sized about 5.0 mm was 75.2% of monad and 24.8% of dyad, and those about 7.5 mm was 10.5% of monad, 87.8% of dyad and 1.7% of tetrad (Table 1) in *P. amabilis*. Therefore most of pollen mother cells (PMCs) at flower bud development stages ranged from 5.0 to 7.5 mm in size must be undergoing meiosis and major PMCs at the stage of flower buds sized about 5.0 mm must be before meiosis Ⅱ in meiotic progression. The results were similar with those of Akutsu *et al.* (2007)'s report that putative 2n pollen grains were formed by treating PMCs in metaphase I to metaphase Ⅱ and the N_2O treatment was most effective in obtaining 2n pollen when administered during metaphase I in lilies. Accordingly, establishing relationship between bud size and meiotic stage through simply sporad observation might be a useful strategy for unreduced male gamete induction needed for developing an efficient sexual polyploidization breeding scheme in *Phalaenopsis*.

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