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Induction of Unreduced Gamete in *Phalaenopsis* by N_2O Treatments

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We attempted to induce unreduced gamete of *Phalaenopsis* by arresting the meiotic process with nitrous oxide gas (N_2O). *Phalaenopsis amabilis* and *P. equestris* (2n=2x=38) were treated with nitrous oxide gas under pressure for 0, 24, 48 and 68 hrs. The treatments for 24 and 48 hrs induced unreduced gamete in *P. amabilis* but those for 68 hrs was harmful for flower bud in *P. amabilis*. No 2n pollen was obtained in *P. equestris*. Thus, the treatment with N_2O may provide a new approach for *Phalaenopsis* breeding.

Key words: Nitrous oxide, *Phalaenopsis*, unreduced gamete

INTRODUCTION

In recent years, the use of numerically unreduced (2n) gametes has received considerable attention in breeding both auto– and allopolyploid crops (Mariani and Tavoletti, 1992; Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003; Barba–Gonzalez *et al*., 2006). Jensen (1974) reported that 2n gametes are spontaneously produced through laborious process, and there is hardly any method available for inducing them when required. In order to overcome this difficulty, it would be essential to develop the methods of 2n gamete induction in desirable genotypes. Although there are reliable methods available for doubling the chromosome numbers in somatic cells, hardly any serious effort has been made in the past to induce 2n gametes in plants through chemical agents. Unlike somatic chromosome doubling, where the process of mitosis is disrupted through the so–called "spindle inhibitor", induction of 2n gametes requires the modification of meiosis in such a way that restitution nuclei are formed. As a consequence, instead of forming n micro– or megaspores, meiotic nuclear restitution should lead to the formation of 2n spores. This requires the disruption of nuclear as well as cytoplasmic divisions that occurs during the process of meiosis. Of the many chemical agents that are known to affect mitosis, *i.e*., colchicine, oryzalin, trifluralin and nitrous oxide (N_2O), N_2O is used as a gas, whereas all other chemicals are used as aqueous solutions (Barba–Gonzalez *et al*., 2006). Östergren (1954) first introduced N_2O to act as a spindle inhibitor.

Nitrous oxide gas (N_2O) was applied to seedlings and zygotes of many crops as a polyploidizing agent instead of colchicines treatment (Östergren, 1954, 1957; Nygren, 1955; Zeilinga and Schouten, 1968; Dvorak *et al*., 1973; Taylor *et al*., 1976; Berdahl and Barker, 1991; Kato, 2002; Kitamura *et al*., 2009). Arresting meiosis with N_2O induces 2n pollen in tulips, and triploids can be formed by crossing diploids with N_2O treated pollen (Okazaki *et al*., 2005). N_2O is also useful for polyploidy breeding in lilies where the optimal timing for induction of diploid pollen is at metaphase I (Akutsu *et al*., 2007). Treatment of interspecific lily hybrids with N_2O gas induced 2n pollen and partially restores pollen fertility (Barba–Gonzalez *et al*., 2006). Thus, N_2O treatment is a new approach for analysis of chromosomes and production of diploid pollen in plant breeding. In the present study we developed a method for induction of unreduced gamete by N_2O treatment in *Phalaenopsis*.

MATERIALS AND METHODS

Plant materials

Phalaenopsis amabilis and *P. equestris* (2n=2x=38) were used in this experiment. Plants were cultivated in 10 cm diameter pots in a greenhouse at the Horticulture Technology Center, National Chiayi University, Chia–yi, Taiwan (R.O.C.).

Chromosome sample preparation and Giemsa staining

Growing root tips were pretreated with 2 mM 8–hydroxyquinoline (Sigma–Aldrich, St. Louis) or 0.1% colchicine (Sigma–Aldrich) solution on a rotary shaker (100 rpm) for 5 h at 20˚C, and were then fixed in 95% ethanol–acetic acid (3:1 by vol.) for 24 h at 4˚C. Samples were digested with 1% (w/v) cellulase (Sigma–Aldrich) and 0.5% (w/v) pectinase (Fluka) in 10 mM of citrate buffer (Pedrosa *et al*., 2001). The digested samples were mounted on a slide glass with a droplet of 10% Giemsa (Sigma–Aldrich), covered with a cover slide glass, and microscopically analyzed (Axio version Rel.4.5, Germany) with a CCD camera (Cannon A620, Japan).

Verification of meiotic stage and N_2O treatment

The obtained segment was observed by the lactophenol–acid fuchsin solution (Lim *et al*., 2001) squash method to determine the meiotic stage of pollen mother

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cells (PMCs) and the bud length was measured simultaneously. Buds in meiotic stages suited for N_2O treatment were kept on the stems and the other buds were cut off. Thereafter, stems with buds undergoing meiosis, when the flower bud sizes were 4.5–5.0 and 4.0–4.5 mm in *P. amabilis* and *P. equestris*, respetively (Table 1), were immediately treated with N_2O gas at room temperature for 0, 24, 48 and 68 hours in a pressure–tolerant cylinder (20 cm inner diameter, 100 cm long, with silicone rubber; Okazaki *et al*., 2005). The pressure was at 5 atm (N_2O:O_2=5:1). After the N_2O gas treatment, the treated plants were grown in the greenhouse.

Sporad quantification in pollen mother cells (PMCs)

Pollen mother cells were stained with lactophenol–acid fuchsin solution (Lim *et al*., 2001). Pollinia from full–sized flower buds were fixed with a 95% ethanol–glacial acetic acid (3:1 v/v) solution for 24 h, hydrolyzed in 1 N HCl for 1 h at 60˚C, rinsed three times with distilled water, and stained with a lactophenol–acid fuchsin solution. The materials for observation were examined under a microscope (Axio version Rel.4.5, Germany) with a CCD camera (Cannon A620, Japan). One thousand PMCs were categorized according to the number of sporads, especially tetrads, at the end of the division II stage and at least three different plants were used for examination. Pictures were also taken using a digital camera (Cannon A620, Japan).

Capsule setting and seed germination rate

Pollen grains from *P. amabilis* treated by N_2O for 0, 24 and 48 hrs were crossed with *P. amabilis*. Ten flowers were used for each crossing. Capsules were harvested 130 days after pollination (DAP). Two weeks after the pollination, number of capsules was counted to clarify capsule settings. Surviving capsules were counted 130 days after the initiation of each treatment. Seed viability was assessed using Alexander's staining process (Alexander, 1980). The seeds were observed under a microscope and pink and red stained seeds were considered viable.

Three capsules per crossing were surface–sterilized for 20 min in 20% (v/v) Clorox solution and 0.05% Tween 20 and subsequently rinsed 3 times with sterilized distilled water. Seeds were germinated aseptically in 90× 15 mm petri dishes containing 25 mL basal medium supplemented with 2.5 g/L Hyponex #1 (7N–6P2O5–19K2O), 200 mg/L myo–inositol, 2 g/L peptone, 1 g/L ammonium nitrate (NH_4NO_3), 60 g/L Potato, 1 mg/L naphthalene acetic acid (NAA), 0.5 mg/L kinetin, 0.5 mg/L indole–3–butyric acid (IBA), 1 g/L activated charcoal 20 g/L sucrose and 10 g/L agar. The pH value of the media was adjusted to 5.4 with 1 N KOH or HCl prior to autoclave for 15 min at 121˚C. All cultures were incubated at 25±2˚C in a 16 hr photoperiod (cool–white fluorescent light lamps light intensity: 54 μmol $m^{-2}s^{-1}$). Each well in in vitro cultures was examined 30 days after seed sowing under a stereoscopic microscope (Olympus SZX12, Olympus Optical Co. Ltd, Tokyo, Japan). Germinated seeds were counted, and Percentages of seed germination for each treatment were averaged from five grids (1 cm^2) randomly selected on each petri dish. Grids typically contained between 50 and 100 seeds each.

Statistical analysis

This experiment used a completely randomized design (CRD) and used the SAS program for statistical analysis of the data looking at the least significant difference (LSD) multiple range test where $P \leq 0.05$.

RESULTS

Distribution of sporad types of PMCs in different size of flower bud

We observed sporad types of PMCs in different size of flower bud of both species. Therefore, the percentage of Dyad type observed was used to establish the stage to analyze the relationship between bud size and meiotic stage (Table 1). The highest percentage of dyad was observed when the flower bud sizes were 4.5–5.0 mm in *P. amabilis* and 4.0–4.5 mm in *P. equestris*.

Effect of time of N_2O treatments on flower survival

N_2O Treatment for 24 and 48 hrs was not harmful to the flower buds to survive, but that given for 68 hrs was harmful in both species (Table 2).

Table 1. Distribution of sporad types of *P. amabilis* and *P. equestris* observed by the staining of PMCs with lactophenol–acid fuchsin solution

	Flower bud length (cm)	Number (percentage) of PMCs in indicated stages				
		Monad	Dyad	Triad	Tetrad	Total
P. amabilis	0.50	912 (75.2)	301 (24.8)	0 (0)	0 (0)	1213 (100)
	0.73	135 (10.5)	1117 (87.8)	0 (0)	21 (1.7)	1273 (100)
	0.97	0 (0)	20 (1.8)	0 (0)	1080 (98.2)	1100 (100)
	1.20	0 (0)	15 (1.2)	0 (0)	1245 (98.8)	1260 (100)
	1.50	0 (0)	0 (0)	0 (0)	1080 (100)	1080 (100)
P. equestris	0.47	939 (90.7)	96 (9.3)	0 (0)	0 (0)	1035 (100)
	0.50	963 (73.0)	357 (27.0)	0 (0)	0 (0)	1320 (100)
	0.61	80 (7.9)	928 (91.3)	8 (0.8)	0 (0)	1016 (100)
	0.74	0 (0)	464 (28.3)	64 (3.9)	1112 (67.8)	1640 (100)
	0.83	0 (0)	8 (0.7)	72 (6.5)	1032 (92.8)	1112 (100)

Table 2. Effect of time period for treated N_2O on the PMCs in mature flowers of *P. amabilis and P. equestris*

	Time (hours)	No. of flower buds treated	No. of flower buds survived[z]	Percentage of flower buds survived
P. amabilis	0 (Control)	20	20	100
	24	20	20	100
	48	20	20	100
	68	20	0	0
P. equestris	0 (Control)	20	20	100
	24	20	20	100
	48	20	20	100
	68	20	0	0

[z] Data collected 7 days after N_2O treatment

Table 3. Effect of time period for treated N_2O on the PMCs in mature flowers of *P. amabilis* and *P. equestris*

	Time (hours)	Number (percentage) of PMCs in indicated stages				
		Monad	Dyad	Triad	Tetrad	Total
P. amabilis	0 (Control)	0 (0)	0 (0)	0 (0)	1089 (100)	1089 (100)
	24	0 (0)	130 (11.0)	0 (0)	1053 (89.0)	1183 (100)
	48	0 (0)	523 (39.9)	0 (0)	788 (60.1)	1311 (100)
P. equestris	0 (Control)	0 (0)	0 (0)	0 (0)	1053 (100)	1053 (100)
	24	0 (0)	0 (0)	0 (0)	1024 (100)	1024 (100)
	48	0 (0)	0 (0)	0 (0)	1028 (100)	1028 (100)

Table 4. Effect of time of N_2O treatments on seed germination in the crosses of *P. amabilis* (♀) with N_2O treated *P. amabilis* (♂)

Time of N_2O treatment (hours)	No. of pollinated flowers	No. of capsules set (%)[z]	Seed viability (%)	Seed germination (%)[y]
0 (Control)	10	10 (100)	85.7 a[x]	67.6 a
24	10	8 (80)	80.9 a	62.6 b
48	10	4 (40)	59.7 b	57.2 c

[z] Data collected 14 days after pollination
[y] Data collected 30 days after seed sowing
[x] Mean separation within columns by LSD multiple range test at $P \leq 0.05$

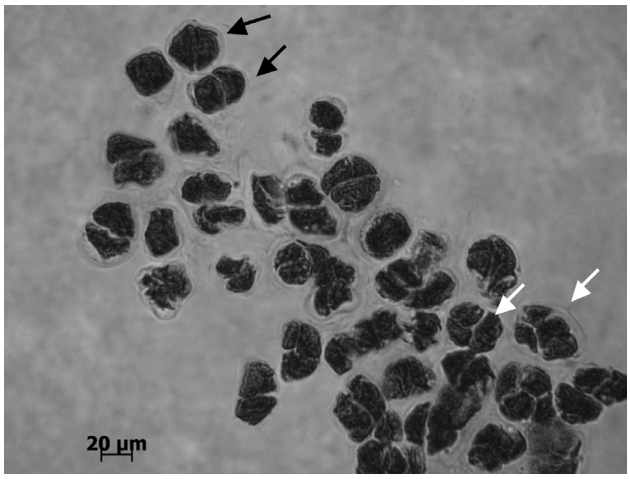

Fig. 1. Distribution of microspore in *P. amabilis* (2n=2x=38) when the plants were exposed to N_2O gas at 0.5 cm flower bud stage. Dyad (indicated by black arrows; 21.6× 16.1 μm); Tetrad (indicated by white arrows; 17.2× 12.4 μm). Scale: 20 μm.

Influence of N_2O on meiosis pollen mother cell (PMCs)

N_2O treatments for 24 and 48 hrs resulted in 11 and 40% dyad (unreduced gamete) of PMCs, respectively in *P. amabilis*, but no dyad was observed in control (Table 3). The dyad pollen size was about 1.3 times larger than the size of normal pollen at the end of meiosis (Fig. 1). No dyad was observed in *P. equestris*.

Effect of N_2O on capsule setting, seed viability and seed germination rate

N_2O treatment was not fatal in *P. amabilis* crossed with N_2O treated pollen, but capsule setting, seed viability and seed germination rate decreased by increasing the treatment time (Table 4, Fig. 2).

DISCUSSION

Our results show that N_2O treatments are effective to increase the percentages of dyad (unreduced gamete)

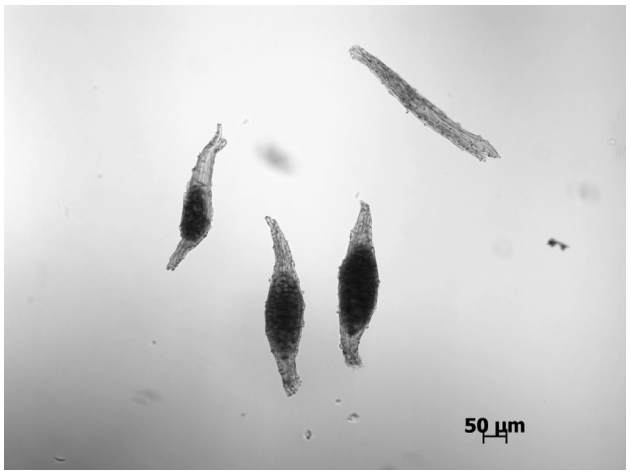

Fig. 2. Effect of N_2O on *P. amabilis* seed viability. Viable seeds were stained and unviable seeds were not. Scale: 50 μm.

and pollen grain size (which is related to DNA content or ploidy level) in *P. amabilis*. N_2O has been used previously in tulips (Okazaki *et al*., 2005), lilies (Barba–Gonzalez *et al*., 2006; Akutsu *et al*., 2007) and *Begonia* (Dewitte *et al*., 2010) to induce unreduced gametes and to restore fertility in sterile genotypes. It has been shown in these cases that the most useful time to start the treatments is when the pollen mother cells proceed through meiosis, but the efficiency of the treatments may vary dependent on the exact stage of meiosis. In contrast with *Begonia*, both tulips and lilies have a successive type of meiosis, where cytokinesis occurs after both meiosis I and II. In our study, *P. amabilis* was treated with N_2O when the flower bud size was consistent with the initiation of meiosis (about 4.5–5.0 mm), although the exact meiotic stage was not monitored during treatment. Because the anthers at the meiotic stage are still very small and flowers may be easily harmed and become useless when anthers are removed to check the meiotic stage. Good timing of the treatments is critical to disturb meiosis and induce unreduced gamete, as meiosis within the genotypes used was limited to bud sizes of 4.5–5.0 mm. In all cases, sporad types were observed approximately 14 days after treatments. Detailed studies on the exact mechanism of N_2O mediated chromosome doubling during meiosis are limited, but it is suggested that in both mitosis (Östergren, 1954; Kihara and Tsunewaki, 1960; Dvorak *et al*., 1973; Kato and Birchler, 2006) and meiosis (Okazaki *et al*., 2005; Barba–Gonzalez *et al*., 2006; Akutsu *et al*., 2007), N_2O disrupts the spindle mechanism. In *Lilium*, Barba–Gonzalez *et al*. (2006) showed that N_2O fumigation produced mainly first division restitution (FDR) gametes, which indicates a disruption of meiosis I. Kitamura *et al*. (2009) showed in *Lilium* that microtubules were depolymerised during metaphase I, which prevented chromosomes from moving to the poles.

The present study demonstrates that N_2O gas can successfully increase the frequency of dyad (unreduced gamete) in *P. amabilis* similar to Chen and Chin (2010)'s report that mitotic inhibitor increased the frequency of dyad *in Phalaenopsis* Sogo Yukidian 'V3' and *P*. Tai Lin Redangel 'V31', but for *P. equestris* further studies are necessary to find suitable time period of the treatments for induction of 2n pollen. The method of N_2O treatment can reduce the time required to obtain 2n pollen and is not harmful to human and plant, unlike use of 2n pollen through tetraploidization of diploids by colchicine treatment or other mitotic inhibitors. Van Tuyl *et al*. (1989) reported that polyploids obtained via colchicines treatment in lilies did not flower for long time because of their slow growth. In addition, the harmful effect of colchicines made it ineffective in doubling the chromosome in Oriental hybrid lilies.

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