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Application of Nitrous Oxide Gas as a Polyploidizing Agent in Tulip and Lily Breeding

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ABSTRACT

Nitrous oxide has been successfully applied to zygotes as a polyploidizing agent in various crops. More recently nitrous oxide treatments have been applied at male gamete formation resulting in production of diploid gametes in tulips and lilies. Additionally this treatment can be used to overcome pollen sterility of interspecific hybrids via polyploidization of archesporial cells in developing anthers. This paper provides a review of some of the literature and results of our experiments using nitrous oxide for chromosome doubling of gametes and zygotes, as well as pollen mother cells to overcome pollen sterility of the interspecific hybrids. These methods have important implications for lily and tulip breeding.

Keywords: chromosome doubling, diploid male gamete, hybrid sterility, interspecific hybridization, lilies, nitrous oxide, polyploidy, tulips, zygote

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INTRODUCTION

In ornamental plant breeding, polyploidization is a useful tool for creating desirable characteristics such as flowering longevity, deep flower color, larger flowers and resistance to physiological disorders (Arisumi 1964; Takamura and Miyajima1996; Ketsa *et al*. 2001; Okazaki and Hane 2005; Liu *et al*. 2007). In Easter lily, tetraploid forms have larger (but fewer) flowers, and thicker leaves, but are later flowering than their diploid counterparts. There is an increased number of flowers in progeny obtained from crosses between tetraploid individuals. In addition, greater variation was observed in populations of tetraploid seedlings than in diploid populations (Emsweller and Uhring 1960). In Asiatic hybrid lilies, the periclinal chimera form (4×/2×) is effective for generating resistance against leaf scorch (Okazaki and Hane 2005). Thus, it is considered advantageous to breed lilies at the triploid/tetraploid level in Easter lily and Asiatic hybrid lily. Indeed, tetraploid Asiatic hybrid lilies have been introduced to commerce (McRae 1987).

Polyploidy is often used to restore fertility in sterile interspecific hybrids in which there is no homology between the two sets of parental genomes. Somatic polyploidization produces amphidiploids with homologous chromosomes coming from autosyndetic chromosome pairs of one parental species to overcome hybrid sterility (Asano 1982; Lim *et al*. 2000; Nimura *et al*. 2006). Naturally occurring and induced formation of 2n gametes from aberrant meiosis, i.e. FDR (first division restitution) and SDR (second division restitution), occurs in interspecific hybrids. These 2n-gametes can be used for the production of sexual progeny either through crossing or selfing (Asano 1984, Lim *et al*.; 2000, 2001; Van Tuyl and Lim 2003; Barba-Gonzalez *et al*. 2004; Khan *et al*. 2009). Thus, techniques such as application of nitrous oxide for inducing polyploidy are important for both inducing polyploidy and for restoring fertility following interspecific hybridization of ornamental plants.

The traditional method of producing polyploids was to double the chromosomes using a polyploidizing agent such as colchicine (Asano 1982; Lu and Bridgen 1997), amiprophos-methyl (Nimura *et al*. 2006), and oryzalin (Van Tuyl *et al*. 1992; Burge *et al*. 2008). Nitrous oxide (N_2O) has been applied to zygotes, seedlings and pollen mother cells (PMCs) as a polyploidizing agent in lieu of colchicine treatment. The toxicity of colchicine means there has been considerable interest in using N_2O treatments for this purpose. Nitrous oxide is suitable for treating organs inside tissues because the gas can simply permeate to reach the tissues of interest, e.g., developing microspores within tulip bulbs (Östergren 1954). Additionally the gas is expected to be rapidly dissipated from treated tissues after the pressure is released, thereby preventing further harmful after-effects. For these reason nitrous oxide has been applied as a polyploidizing agent to zygotes in several crops and is how 2n

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Invited Mini-Review

Table 1 N_2O treatments to zygotes and seedlings in some species.

Species / Crops	Treated materials	Hours/days to treatment after crossing	Pressure of N_2O (atm)	Duration of N_2O (h)	References	Remarks
Melandrium	Zygote	18-24 h	2, 5, 10	4-16	Nygren 1955	No effect of the 16-48 h treatment
Crepis	Zygote	7-11 h	10	4-6	Östergren 1954	
Phalaris	Zygote	Panicle just completed flowering	10	4-12	Östergren 1957	
Triticum dicoccum	Zygote	24 h	3,6	10, 15	Kihara and Tsunewaki 1960	No effect of the 5 h treatment
Wheat	Zygote	24 h	4	24	Dvorak *et al*. 1973	
Barley	Zygote	6 h	4	24	Dvorak *et al*. 1973	
Trifolium	Zygote	24 h	6	24	Tayor *et al*. 1976	
Russian wildrye	Zygote	20 h	5	24	Berdahl and Barker 1991	
Maize	Zygote	30-36 h	8-9	20	Kato and Birchler 2006	
Tulips	Zygote	6-10 days	5-7	24	Zeilinga and Schouten 1968	Necrosis at higher pressure
Lilies	Zygote	10-13 days	6-8	48-78	Okazaki *et al*. unpublished data	
Wheat	Haploid plants	Small seedlings prior to transplanting	6	24-48	Hansen *et al*. 1988	
Maize	Haploid plants	6-leaf stage seedlings	6	48	Kato 2002	

pollen can be produced in tulips by arresting meiosis in the anthers with nitrous oxide (Okazaki *et al*. 2001; Okazaki *et al*. 2005). In some cases N_2O treatment was highly effective at inducing polyploidy. This paper provides a review of recent work on N_2O treatment of zygotes and male gametes, and provides comments on the experimental conditions necessary for successful polyploid production when using N_2O.

APPLICATION TO ZYGOTES

Östergren (1944) discovered that N_2O could induce c-mitosis. He observed c-mitosis in root tips of peas and onion treated with N_2O, finding that peas are much more sensitive than onion to N_2O. He subsequently applied this approach to zygotes of *Crepis* (Östergren 1954) and *Phalaris* (Östergren 1957). To date N_2O has been applied as a polyploidizing agent to zygotes in various crops as listed in **Table 1** (Östergren 1954, 1957; Nygren 1955; Zeilinga and Schouten 1968; Dvorak *et al*. 1973; Taylor *et al*. 1976; Berdahl and Barker 1991; Kato and Birchler 2006). For successful treatment of zygotes, it is necessary to first determine optimal conditions for the N_2O application, i.e., when to treat plants (timing of application), gas pressure and duration of treatment.

A detailed knowledge of fertilization and early embryonic development is needed prior to N_2O application. When flowers with zygotes undergoing the first mitosis are treated with N_2O, the resulting embryos will become fully polyploid, while later treatment may result in chimaeras. In some cases chimaeric sectors of zygotes may be eliminated as a result of competition between cells with different ploidy levels. Thus the first mitotic division is the optimal timing for N_2O treatment of zygotes. In the Poaceae, the first zygotic mitosis takes place shortly after fertilization. Based on this knowledge, plants were treated at 6 hours after pollination in barley (Dvorak *et al*. 1973), at 24 hours in wheat (Kihara and Tsunewaki 1960) and 30–36 hours in maize (Kato and Birchler 2006). Similarly in species such as *Crepis* (Ostergren 1954) and *Melandrium* (Nygren 1955), N_2O treatment shortly after pollination is optimal for polyploid production.

Other species have different processes of pollination and fertilization. For best results tulips should receive N_2O treatment 6-10 days after pollination (Zeilinga and Schouten 1968). Pollen tubes of lilies take at least 48 h to reach the ovary. Based on this observation and the result obtained in tulips, we initially attempted to treat lilies with N_2O 1–7 days after pollination. A small number of polyploid plants were obtained with treatment 7 days after pollination but no polyploid plants were obtained from flowers treated 1–5 days after pollination. When we treated flowers 10–13 days after pollination we obtained a surprisingly high frequency of polyploid plants and from some capsules all of the seed-derived plants were polyploid (**Fig. 1**). This indicates that the first division of the fertilized eggs of lilies takes place 10–13 days after pollination, which is in agreement with the cytological observations of Janson *et al*. (1995) and Ikeda *et al*. (2003). In our experiments we were unable to maintain precise temperature control during growth and N_2O treatment, and the optimal time for treatment will probably vary with temperature.

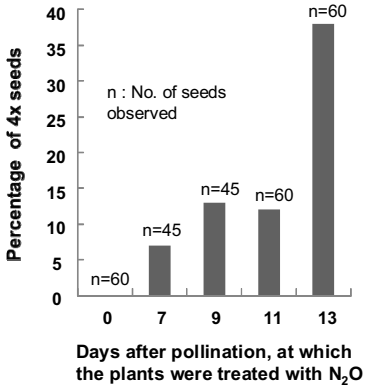

Fig. 1 Production rate of 4× seeds obtained by treating the plants derived from the cross of 'Regata' × 'Mona' of Asiatic hybrids the 48h • 6atm N_2O treatment.

The duration of N_2O treatment and the pressure needed may vary between species. There is evidence that the sensitivity to N_2O varies among species, breeding lines and even cultivars (Östergren 1954; Dvorak *et al*. 1973; Kato and Birchler 2006). For example, pea seedlings showed c-mitosis in root tips treated with N_2O at 1 atm for 9 h, whereas a 1 atm treatment applied to onions did not result in c-mitosis but the 4 atm treatment worked (Östergren 1954). For Asiatic hybrid lilies, when flowering plants, 10–13 days after pollination, were treated with N_2O for 48 or 72 h, at pressures of 3, 6 or 8 atm, the 6–8 atm treatments resulted in polyploids but the 3atm treatment had no observed effect. There was no significant difference between the 48 and 72 h treatments (unpublished data). This indicates that the critical period for N_2O treatment for Asiatic hybrid zygotes is somewhere between 48 and 72 h for both 6 and 8 atm pressures. Wheat plants were treated with N_2O for 5, 10, 15 h, at pressures of 3 and 6 atm for each treatment duration; the 10 and 15 h treatments (for both 3 and 6 atm pressures) resulted in polyploid plants but the 5 h treatment did not (Kihara and Tsunewaki 1970). Kihara and Tsunewaki (1970) also found that the treatment of 10 h at 3

atm resulted in aneuploids with close to the diploid chromosome number in addition to producing tetraploids. Kato and Birchler (2006) treated zygotes of maize inbred lines with N_2O for 20 h at 6-10 atm. They demonstrated that the 8-9 atm treatments were better than 6 atm.

APPLICATION TO PMC UNDERGOING MEIOTIC DIVISION

Treating plants undergoing meiotic division with N_2O has been shown to be effective for chromosome doubling of male gametes. The N_2O treatment arrests meiosis leading to formation of 2n gametes (Okazaki *et al*. 2001, 2005). Other chemicals induce mitotic polyploidization by arresting cell division whereas N_2O treatment of PMC can result in FDR (first division restitution) and SDR (second division restitution) mechanisms producing numerically 2n gametes. The resulting 2n gametes are functional and may be used for crossing. In fact, 2n gametes obtained through N_2O treatment have resulted in polyploid plants. To date however, application of the N_2O technique to meiosis is limited to a few cases; tulips (Okazaki *et al*. 2005), lilies (Barba-Gonzalez *et al*. 2006; Akutsu *et al*. 2007) and begonias (Dewitte *et al*. 2010).

In their histological study on meiotic PMC of lilies treated with N_2O gas, Kitamura *et al*. (2009) reported that microtubules were effectively depolymerized; this prevented chromosomes from moving to the poles, resulting in chromosome retention in the center of N_2O-treated cells. Cell plate formation took place without delay, yielding one daughter cell with a diploid genome and another daughter without chromosomes. Thus the 2n male gamete is produced in cells undergoing meiotic metaphase (**Fig. 2**). According to this mechanism for formation of 2n pollen, optimal timing for N_2O treatment to achieve meiotic polyploidization must be meiotic metaphase I in PMC. To confirm which meiotic stage is optimal for induction of 2n pollen, plants with buds at different meiotic stages were treated with N_2O for 24 h. A few 2n pollen grains were induced using plants with anthers in prophase I, whereas a mixture of n, 2n and aneuploid pollen grains were produced using plants with PMC predominantly at meiotic metaphase (Akutsu *et al*. 2007).

For tulips, meiosis in anthers occurs inside the bulbs from mid- to late-October. When meiosis in anthers reached metaphase I, other bulbs of the same clones were treated with N_2O for 24–48 h. Most of the treated plants produced a mixture of n, 2n and aneuploid pollen grains (Okazaki *et al*. 2005). In begonias, N_2O and trifluralin were applied to small floral buds to induce 2n pollen formation with N_2O treatments giving better results (Dewitte *et al*. 2010). The begonia plants were treated with N_2O when the bud size was consistent with the initiation of meiosis (about 4 mm), although the treated floral buds were too small to monitor what meiotic stage was underway in the anther at the time of the N_2O treatment.

Treating buds with PMC at meiotic metaphase has proven optimal for induction of 2n pollen in tulips and lilies. Since meiosis is completed relatively quickly, the optimal period for treatment is limited to only a very short time. It is important to identify which meiotic stage is underway at the time of the N_2O treatment. Floral bud length is a good criterion to identify the meiotic stage of PMC in lilies because there is a rough correlation between bud size and the developmental stage (Tayor and McMaster 1954; Sano and Tanaka 2005). In the case of *L. longiflorum*, the floral buds shorter than *ca*. 10 mm are in the proliferating stage of premeiotic PMC and buds of *ca*. 10–22 mm are in meiotic prophase I. Buds of *ca*. 23–25 mm are undergoing meiotic division with tetrads present for a short time (Taylor and McMaster 1954). Histological studies have confirmed that in Asiatic lily hybrids prophase I is observed in the *ca*. 15 mm floral buds and metaphase I is found in *ca*. 20 mm floral bud (Akutsu *et al*. 2007).

It is known that when tulips and lilies undergoing meiotic division are treated with N_2O, a mixture of n, 2n and aneuploid pollen grains are produced (Okazaki *et al*. 2005; Akutsu *et al*. 2007). This is due to low synchronization of PMCs in meiotic metaphase. If the N_2O treatment is applied to PMC which are completely synchronized at meiotic metaphase, the resulting pollen would be 100% 2n pollen. To achieve a high 2n pollen production rate, it is important to monitor meiotic stages of the plants and to choose plants synchronized in meiotic metaphase at the time of the N_2O treatment.

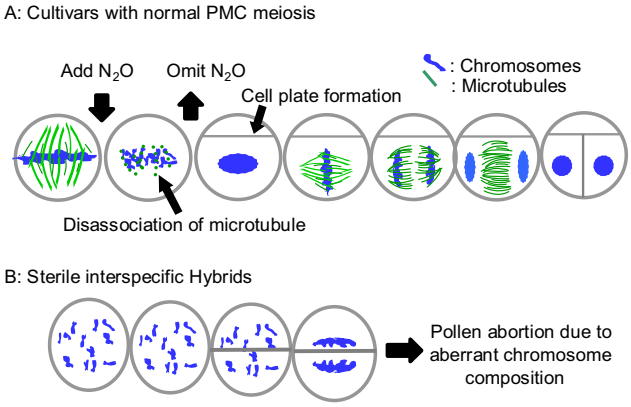

Fig. 2 The mechanism by which 2n pollen is produced with N_2O in normal meiotic stage and sterile hybrids produce aneuploid pollen with or without N_2O treatment.

APPLICATION TO ARCHESPORIAL CELL UNDERGOING MITOTIC DIVISION IN ANTHER

Optimal timing for mitotic polyploidization of archesporial cells in anther

In addition to meiotic polyploidization with N_2O, we found that the N_2O treatment is effective for mitotic polyploidization of male archesporial cells by treating plants with archesporial cell proliferating stage (Nukui *et al.* 2011). The differentiation of male archesporial cells starts in 1 mm floral buds of Asiatic hybrids and the archesporial cells continue mitotic division in the *ca*. 7 mm floral buds. At this stage, the N_2O treatment to Asiatic hybrid 'Regata' induces mitotic polyploidization of archesporial cells so that giant pollen grains are obtained (**Fig. 3**). The mixture of normal and giant pollen is thought to include n, 2n and aneuploid pollen grains (**Fig. 3B**). Since it is known that N_2O affects cells in active cell division, it is essential to identify cells having high mitotic index. As mentioned previously, there is a rough correlation between developmental stages of PMC and bud lengths, and this size-based criterion is useful for identification of optimal timing for N_2O treatments. On this basis, we treated plants with various sized floral buds to

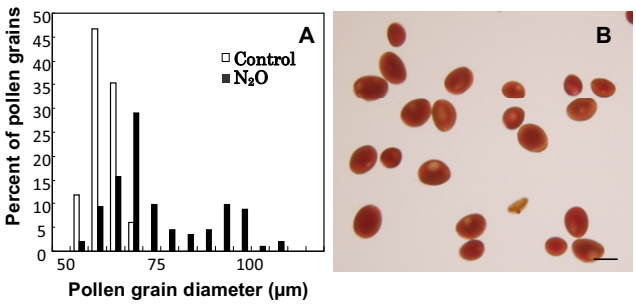

Fig. 3 Size distribution and appearance of pollen grain in diploid Asiatic hybrid 'Regata'. (**A**) Size distribution of control pollen (shaded bars) and pollen obtained from plant treated with N_2O gas for 48 h (open bars). (**B**) Mixed pollen grains with different sizes obtained from the N_2O-treatment to the premeiotic PMC stage. Bar = 50 μm.

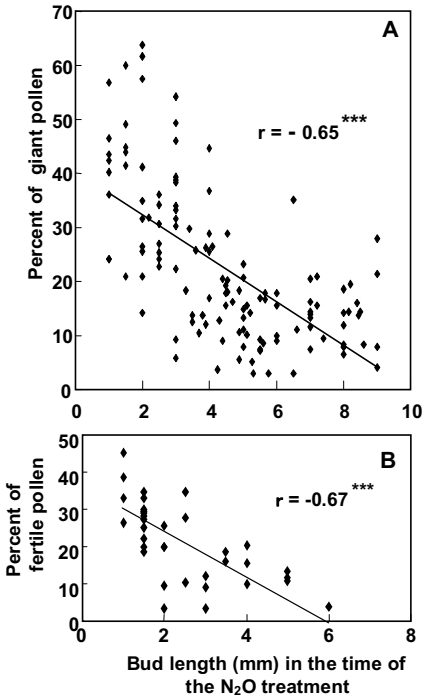

Fig. 4 Correlation of floral bud length in the time of N_2O treatment and percent of giant pollen obtained with N_2O. (**A**) Asiatic hybrid 'Regata'. (**B**) Fertile pollen restored in sterile OT hybrid 'Yelloween'. ***: Significant at 0.1% level.

identify the optimal stages for inducing mitotic polyploidization of male archesporial cells. The optimal timing for N_2O treatment for lilies is genotype dependent as follows; 1 to 4 mm for Asiatic hybrid lilies (**Fig. 4A**), and 2 to 6 mm for Oriental hybrid lilies and LA hybrid lilies (Nukui *et al.* 2011).

Overcoming of hybrid sterility

Lily breeders must work with hybrid sterility in LA hybrids as well as OT, OA and LO hybrids. In the latter three hybrids, hybrid sterility is a more serious problem. Barba-Gonzalez *et al*. (2006) reported that the treatment of sterile interspecific lily hybrids with N_2O gas successfully overcame pollen sterility. We found that by treating 1-6 mm buds of the completely sterile OT hybrid 'Yelloween' with N_2O gas we successfully overcame pollen sterility (**Fig. 4B**). The optimal bud size was 1-4 mm. This bud size corresponded to the archesporial cell proliferating (Nukui *et al*. 2011).

Barba-Gonzalez *et al*. (2006) showed that the N_2O treatment successfully induced fertile pollen through FDR. However, the FDR mechanism by which hybrid sterility can be restored is not explained by the findings of Kitamura *et al*. (2009). N_2O inactivates spindle formation in meiotic division and induces 2n male gametes by preventing the movement of the sister chromosomes toward the opposite poles. In the case of interspecific hybrids where the chromosomes are scattered in the cytoplasm due to no homology between the two parental genomes, the cell plate divides the chromosomes unequally to produce aneuploid daughter cells with or without the N_2O treatment (**Fig. 2**). This suggests that N_2O treatment at the meiotic division does not lead to chromosome doubling in a daughter cell through FDR. Although Barba-Gonzalez *et al*. (2006) do not discount the possibility that restoration of hybrid sterility might be due to pre-meiotic chromosome doubling of PMC, they suggested that N_2O treatment produced mainly FDR gametes in the sterile OA hybrids, based on the appearance of recombinant chromosomes in N_2O derived progeny. Our study disagrees with the cytological FDR mechanism of action of N_2O treatment for overcoming hybrid sterility.

Table 2 Summary of N_2O treatments to male gamete formation stage.

Crops	The optimal stage for N_2O treatment	Production of 2n pollen	Overcoming hybrid sterility
Meiotic polyploidization			
Tulips	Metaphase I of PMCs	Yes	No
Lilies	Metaphase I of PMCs	Yes	No
Mitotic polyploidization			
Lilies	Archesporial cells	Yes	Yes

Kato (2002) suggested that it might be possible to overcome hybrid sterility by applying N_2O to the floral primordial stage that undergoes mitotic division. This idea is in agreement with our results.

THE COMPARISON BETWEEN MEIOTIC AND MITOTIC TREATMENTS

Chromosome doubling by N_2O treatment of floral buds has two optimal stages. One is at meiotic division and the other is the archesporial cells proliferation stage (**Table 2**). For the N_2O treatment at meiotic metaphase I, 18-23 mm floral buds are suitable in Asiatic hybrid lilies. The larger floral buds of OH hybrids means the optimal bud size for treating OH hybrids is bigger than for AH hybrids and more variable depending on genotype. Floral buds of 20-34 mm were optimal in OH hybrids. These size-based criteria are useful for identifying the stage to apply the N_2O treatment but are not absolute because both floral bud development and PMC development are affected by environmental and/or cultural conditions. Since the meiotic division is over relatively quickly, the optimal treatment time is limited to a short period. PMCs at meiotic metaphase I stage were better synchronized for cell division, compared to the archesporial cell proliferating stage. Consequently N_2O treatment at the PMC meiotic division stage can achieve a higher production rate of diploid male gametes than treating at the archesporial cell proliferating stage. The N_2O treatment at the archesporial cell proliferating stage is useful for both overcoming hybrid sterility and for mitotic diploidization of male gametes of fertile cultivars. By comparison treatment at meiotic metaphase I is only applicable to polyploidization of male gametes. Handling the plants at the pre-meiotic stage is easier than at the meiotic division stage because of the smaller size of the plant at the archesporial cell proliferating stage. Additionally there is a longer developmental window during which N_2O treatment may be effectively applied in the archesporial cell proliferating stage buds. It is possible to choose between the two N_2O treatment methods depending on given cultivars and experimental aims.

Finally, the treatment with N_2O has proven useful for the production of 2n pollen in tulips (Okazaki *et al*. 2005) and lilies (Akutsu *et al*. 2007) as well as for overcoming hybrid sterility in lilies (Barba-Gonzalez *et al*. 2006; Nukui *et al*. 2011) and begonias (Dewitte *et al*. 2010). Additionally N_2O treatment of lily zygotes resulted in a high rate of production of tetraploid seedlings (unpublished data). It is suggested that the potential usefulness of these techniques should be tested in a greater range of ornamental crops.

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