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 Miyajima 1996; 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 (4x/2x) 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 (N2O) 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 N2O 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 reasons nitrous oxide has been applied as a polyploidizing agent to zygotes in several crops and is how 2n
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 N₂O treatment was highly effective at inducing polyploidy. This paper provides a review of recent work on N₂O treatment of zygotes and male gametes, and provides comments on the experimental conditions necessary for successful polyploid production when using N₂O.

**APPLICATION TO ZYGOTES**

Östergren (1944) discovered that N₂O could induce c-mitosis. He observed c-mitosis in root tips of peas and onion treated with N₂O, finding that peas are much more sensitive than onion to N₂O. He subsequently applied this approach to zygotes of *Crepis* (Östergren 1954) and *Phalaris* (Östergren 1957). To date N₂O 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 best results tulips should receive N₂O shortly after pollination is optimal for polyploidy. This paper provides a review of the duration of treatment.

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APPLICATION TO ZYGOTES

Östergren (1944) discovered that N₂O could induce c-mitosis. He observed c-mitosis in root tips of peas and onion treated with N₂O, finding that peas are much more sensitive than onion to N₂O. He subsequently applied this approach to zygotes of *Crepis* (Östergren 1954) and *Phalaris* (Östergren 1957). To date N₂O 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 best results tulips should receive N₂O shortly after pollination is optimal for polyploidy. This paper provides a review of the duration of treatment.

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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 N₂O 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 N₂O has been shown to be effective for chromosome doubling of male gametes. The N₂O treatment arrests meiosis leading to formation of 2n gametes (Okazaki et al. 2001, 2005). Other chemicals induce mitotic polyploidization by arresting cell division whereas N₂O 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 N₂O treatment have resulted in polyploid plants. To date however, application of the N₂O 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 historical study on meiotic PMC of lilies treated with N₂O 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 N₂O-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 N₂O 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 N₂O 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 N₂O 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, N₂O and trifluralin were applied to small floral buds to induce 2n pollen formation (Fig. 2). According to this mechanism for formation of 2n pollen, optimal timing for N₂O 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 N₂O 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).

In addition to meiotic polyploidization with N₂O, we found that the N₂O 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 N₂O 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 N₂O 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 N₂O treatments. On this basis, we treated plants with various sized floral buds to meiotic division are treated with N₂O, 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 N₂O 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 N₂O 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 N₂O, we found that the N₂O 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 N₂O 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 N₂O 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 N₂O treatments. On this basis, we treated plants with various sized floral buds to
identify the optimal stages for inducing mitotic polyploidization of male archesporial cells. The optimal timing for N2O 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 N2O gas successfully overcame pollen sterility. We found that by treating 1-6 mm buds of the completely sterile OT hybrid ‘Yelloween’ with N2O 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 N2O 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). N2O 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 N2O treatment (Fig. 2). This suggests that N2O 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 N2O treatment produced mainly FDR gametes in the sterile OA hybrids, based on the appearance of recombinant chromosomes in N2O derived progeny. Our study disagrees with the cytological FDR mechanism of action of N2O treatment for overcoming hybrid sterility.

Kato (2002) suggested that it might be possible to overcome hybrid sterility by applying N2O 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 N2O treatment of floral buds has two optimal stages. One is at meiotic division and the other is the archesporial cell proliferation stage. The optimal timing for N2O treatment at the archesporial cell proliferating stage is 18-23 mm floral buds for 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 N2O 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 N2O 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 N2O 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 N2O treatment may be effectively applied in the archesporial cell proliferating stage buds. It is possible to choose between the two N2O treatment methods depending on given cultivars and experimental aims.

Finally, the treatment with N2O 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 N2O 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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