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Nitrous oxide (N_2O) induces 2*n* gametes in sterile F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids and leads to intergenomic recombination

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Summary

Eight different genotypes of the F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids ($2n = 2x = 24$) were treated with nitrous oxide (N_2O) gas under pressure for 24 and 48 hours. At the time of treatment, all plants possessed early meiotic stages in the anthers of the oldest flower buds. The mature flowers from treated plants were monitored for fertility through pollen germination *in vitro* as well as by using them in crosses with diploid Asiatic hybrids ($2n = 2x = 24$) both as male and female parents. In five out of the eight genotypes of OA hybrids there was evidence for the production of *2n* pollen which germinated *in vitro* from either one or both treatments. The *2n* pollen from three genotypes was successfully used in crosses. In two cases, the treated plants were successfully used as female parents which indicated the formation of *2n* (or 2x) egg cells. From an analysis of 41 sexual polyploid progenies obtained from N_2O treated plants it was shown that they were all euploids consisting of 34 triploids ($2n = 3x = 36$) and seven tetraploids ($2n = 4x = 48$). A detailed cytological analysis of 12 progeny plants through genomic *in situ* hybridization (GISH) proved that N_2O had induced first division restitution gametes in most cases and in two cases they produced gametes through indeterminate meiotic restitution. There was evidence for intergenomic recombination in three cases.

Introduction

In recent years, the use of numerically unreduced (*2n*) gametes has received considerable attention in breeding both auto- and allopolyploid crops (Mariani & Tavoletti 1992; Bretagnolle & Thompson 1995; Ramanna & Jacobsen 2003). For the synthesis of allopolyploids especially, 2*n* gametes can be useful for the following reasons:

1. Sterile F_1 hybrids can be readily used without chromosome doubling if they produce 2*n* gametes.
2. Interspecific recombination can be accomplished because the homoeologous chromosomes are "forced" to pair in diploid interspecific hybrids, e.g. *Lilium* (Karlov et al., 1999; Lim et al., 2003) and *Alstroemeria* (Kamstra et al., 1999).
3. Sexual polyploids can be more effective for generating genetic variation in the F_2 and BC_1 progenies (Ramanna et al., 2003; Barba-Gonzalez et al., in press).

Despite these advantages, however, there is a limitation for routinely using 2*n* gametes in crop breeding. At present, the genotypes that produce 2*n* gametes spontaneously are selected through laborious process, and there is hardly any method available for inducing 2*n* gametes when required. In order to overcome this difficulty, it would be essential to develop methods of

inducing 2*n* gametes in desirable genotypes. Although there are reliable methods available for doubling the chromosome numbers in somatic cells (review by Jensen 1974), hardly any serious attempt has been made in the past to induce 2*n* gametes in plants through chemical agents. Unlike somatic chromosome doubling, where the process of mitosis is disrupted through the so-called "spindle poisons", induction of 2*n* 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 2*n* spores. This requires the disruption of nuclear as well as cytoplasmic divisions that occur during the process of meiosis.

Of the many chemical agents that are known to affect mitosis, viz., colchicine, oryzalin, vinblastine and nitrous oxide (N_2O), among others, the last of these is unique in one respect. Whereas all other chemicals are used as aqueous solutions, N_2O is used as a gas under pressure. Its ability to act as a 'spindle poison' was first introduced by Östergren (1954). A notable advantage of using a gas for treatment is that the toxic effects, if any, can be mitigated by simply removing the tissue from the gas chamber, a process that can not be accomplished when tissues are treated with solutions. For the purpose of somatic chromosome doubling, N_2O has been successfully applied in some plant species such as *Crepis capillaries*; *Phalaris canariensis* (Östergren, 1954, 1957) *Melanrium* (Nygren, 1955); wheat and barley (Tsunewaki, 1962; Dvorak et al., 1973); clover (Giri et al., 1983) and *Psathyrostachys juncea* (Berdahi and Barker, 1991). In many of these cases, in addition to inducing polyploids, N_2O treatment also led to aneuploid production. Cytological explanation for the occurrence of aneuploids has not been provided. In one cytological investigation on the effect of N_2O in *Tradescantia*, Montezuma-De-Carvalho (1973) reported on the inhibition of meiotic spindle at prometaphase – metaphase I stage and gave rise to nuclear restitution. Such cells, on recovery, proceeded to normal second division giving rise to dyads. The consequences of dyad formation were not investigated in this study.

With the aim of inducing 2*n* gametes in completely sterile F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids, whole plants with flower buds, possessing early meiotic stages, were treated with N_2O for different durations. The successful production of 2*n* gametes and sexual polyploids in this experiment are reported and discussed.

Material and methods

Plant material and N_2O treatment

Whole plants with flower buds ranging 0.5 to 1 cm from eight different genotypes of Oriental × Asiatic *Lilium* F_1 hybrids ($2n = 2x = 24$) were placed in a gas chamber and treated with N_2O at a pressure of 6 bars during 0 h (untreated control), 24h and 48h (Table 1) as described by Zeilinga and Schouten (1966). Plants from the different genotypes were not always available to test them under all the different treatments. With two exceptions, 951502–1 and 952400–1, all others (Table 1) were completely sterile when tested during three consecutive seasons. The two exceptions produced low frequencies of 2*n* gametes (Barba-Gonzalez et al. 2004). Asiatic cultivars used were 'Vivaldi' and 'Mont Blanc'. All plants were grown in greenhouses following standard procedures normally used for lily growing.

Fertility

Two criteria were used in order to determine the fertility. a) *In vitro* pollen germination. This was carried out in artificial agar medium containing 100 g sucrose, 5 g bacteriological agar, 20 mg boric acid and 200mg calcium nitrate per litre cultured during 24 h at 25 °C. b) Embryo formation after crossing N_2O treated plants with fertile genotypes. For this, swollen fruits were collected 40–60 days after pollination, submerged in 80% ethanol and flamed for surface sterilization. Swollen ovules generally contained an embryo and when it was possible, the embryo was removed form the ovule and cultured in enriched media (Van Tuyl et al., 1991),

Table 1. Pollen germination percentage (range) per genotype under different N_2O treatments

	N_2O Treatment		
Genotype	0 h	24 h	48 h
951301–5	0	70 (0–95)	–
951502–1	7 (0–30)	15.3 (1–55)	73.75 (55–80)
951914–1	0	0	0
952059–9	0	0	21.3 (15–25)
952400–1	4.5 (0–35)	10.6 (0–30)	36.8 (5–60)
952521–1	1.1 (0–5)	0.5 (0–2)	1.1 (0–4)
962377–1	0	0	–
969023–2	0	5.7 (1–60)	–

otherwise the embryo-sac or the ovule containing the embryo were cultured as such.

In situ *hybridization*

Root tips from the progenies were collected early in the morning and pre-treated in saturated α-bromonaphtalene solution in ice-water overnight; fixed in ethanol acetic acid (3:1) and stored at $-20\,^{\circ}$C until use. The root tips were softened for about 2 h at 37 °C in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase Y23, 0.2% (w/v) cellulase RS and 0.2% (w/v) cytohelicase in 10 m*M* citrate buffer (pH 4.5). Squash preparations were made in a drop of 50% acetic acid; frozen in liquid nitrogen to remove the cover-slip with a razor blade and dehydrated in absolute ethanol and air-dried. Slides were incubated at 37 °C during 1h in RNase A (100 μg/ml); incubated 10 min in pepsin (5 μg/ml), followed by 10 min in paraformaldehyde (4%) at room temperature, between every step the slides were rinsed in 2 × SSC. Slides were dehydrated in 70%, 90% and absolute ethanol during 3 min each and air dried.

Hybridization followed using a mixture consisting of 20 × SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS, 25–50 ng of probe DNA (sonicated genomic DNA (1–10 kb) from the Oriental cultivar 'Sorbonne' labelled with Biotin-16-dUTP by nick-translation according to manufacturer instructions (Roche, Germany)) and 3 μg per slide of autoclaved DNA (100–500 bp) from the Asiatic cultivar 'Connecticut King'. The hybridization mixture was heated at 70 °C for 10 min and then placed on ice for at least 10 min. 40 μl of hybridization mixture were applied to each slide followed by denaturation at 80 °C for 10 min and incubation at 37 °C overnight in a humid chamber. Slides were washed for 15 min at room temperature in 2 × SSC and 30 min in 0.1 × SSC at 42 °C. The probe was detected with Cy3 labelled streptavidin (Amersham Biosciences, UK), and amplified with biotinylated goat-antistreptavidin (Vector laboratories, Burlingame, CA). Chromosomes were counterstained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector Laboratories. Brulingame, CA) was added for its examination under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescent illumination, filter sets of DAPI and Cy3. Images were captured by a Photometrics Sensys 1,305 × 1,024 pixel CCD camera, processed with Genus Image Analysis Workstation software (Applied Imaging Corporation) and sharpened with a 7 × 7 High Gauss spatial filter. DAPI fluorescence was pseudo-coloured in blue and the Oriental DNA probe fluorescence in red. Optimal brightness and contrast were achieved with Adobe Photoshop image processing.

Results

Effect of N_2O treatment on fertility

The eight different genotypes of OA hybrids that were treated with N_2O for durations of 0 h, 24h and 48h, were tested for "fertility" as well as crossability. From our extensive studies on other genotypes of OA hybrids it was established that only when large, well-filled pollen grains are formed in the F_1 hybrids, they represented 2*n* pollen which germinated *in vitro* as well as they were functional when used for crossing. In view of this, the pollen grains were monitored after staining and there was evidence for the presence of 2*n* pollen in variable frequencies in some genotypes (data not included). However, a more reliable criterion was *in vitro* germination of pollen grains. In all cases, there was very little or no pollen germination in the controls (0h treatment in Table 1) whereas considerably high pollen germination in five genotypes that were treated with N_2O (Table 1). In order to calculate the frequencies of pollen germination, only the large and well-filled grains were taken into account and the numerous aborted small structures were ignored. Based on these counts, highest germination percentage was observed in 951301–5 at 24 h treatment duration. The range was calculated on the basis of pollen germination among flowers within a treatment because there was considerable variation among them. Whereas more than 5% of pollen germinated in five genotypes, there was very little or no germination in the other three N_2O treated plants. A dosage effect was observed in N_2O treatment for 24h and 48h when compared to the untreated controls, especially in the case of 951502–1 (Figure 1a and b). However, it was not possible to ascertain whether all the treated flower buds had the same or similar cell division stages at the time of treatment.

All plants that showed 5% or more of pollen germination were used as male parent to be crossed with Asiatic fertile genotypes in order to determine their ability to produce embryos and progenies. Simultaneously, the fertile genotypes were also used as female parents in crosses with Asiatic fertile genotypes. In two genotypes, 951301–5 and 969023–2, embryos were obtained when they were used both as male and female

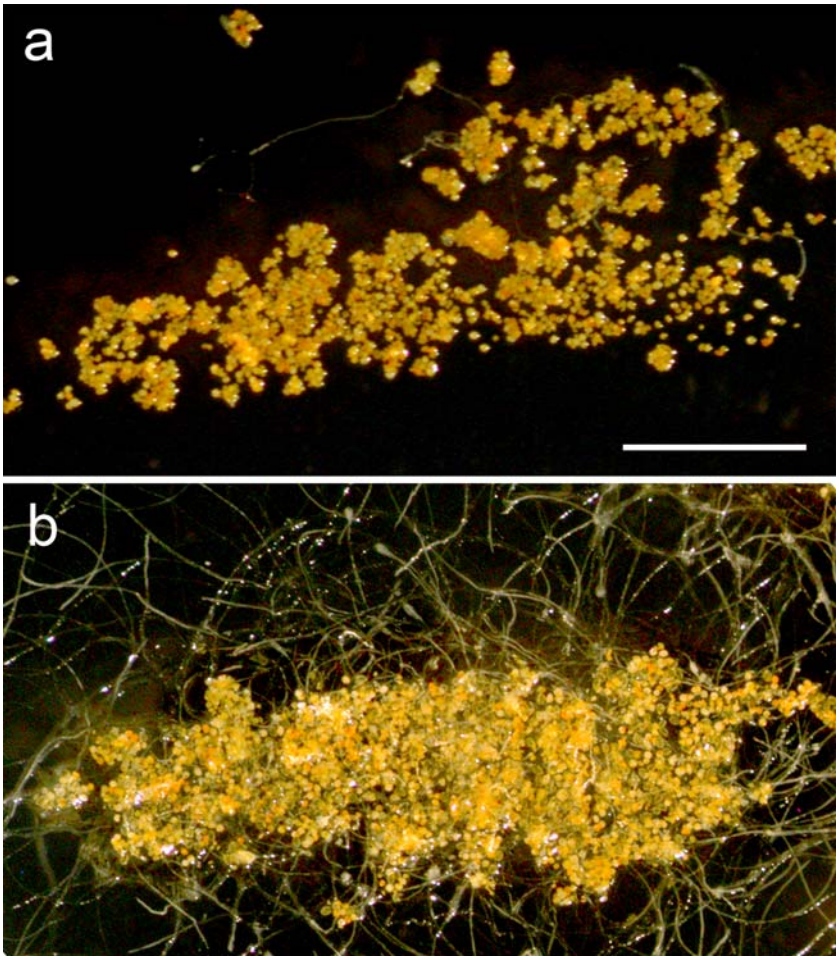

Figure 1. Germination of 2*n* pollen from the OA hybrid 951502–1. (a) Untreated (0 h treatment). (b) After 48 h of N_2O treatment. *Bar*: 0.5 cm.

parent. The other genotype, 951502–1, was successful in producing embryos when used as male parents only (Table 2). In the case of 952400–1 a low frequency of embryos were formed in the control (untreated) plants. 951502–1 and 952400–1 were previously known to produce a low frequency of 2*n* gametes, but in the present case only 951502–1 responded to the N_2O treatments. In view of the high fertility observed in the case of 951301–5, a fairly large number of crosses were made using this genotype in order to utilize the progenies for further analyses.

Ploidy levels and chromosome constitution of progenies derived from N_2O treatment

A total of 41 plants were analysed for their ploidy levels (Table 3). Of these, 29 plants were obtained from crossing N_2O treated 951301–5 as male parent and 12 from using it as female parent. Among the progenies there were 34 triploids ($2n = 3x = 36$) and seven tetraploids ($2n = 4x = 48$). The notable feature was that all had euploid chromosome complements. The seven tetraploid progeny plants were found only when 'Vivaldi', the Asiatic cultivar, was used as the female parent but not in the reciprocal cross. It was concluded that the tetraploid progenies were the result of the functioning of 2*n* eggs of spontaneous origin from 'Vivaldi'. In view of the occurrence of all euploid progeny plants, it was evident that N_2O treated 951301–5 had contributed balanced, $2n = 24$ chromosomes to the progenies in all cases.

The occurrence of euploid progenies from N_2O treated genotypes when used as parents implied that the OA hybrid had contributed 12O + 12A genome

Table 2. Number of embryos obtained (number of pollinated flowers) from crosses of OA hybrids treated during different times with N_2O, with the Asiatic hybrids 'Vivaldi" and 'Mont Blanc'

Genotype	Used as	N_2O Treatment 0 h	24 h	48 h
951301–5	♀	*	32 (10)	–
	♂	*	176 (19)	–
951502–1	♀	0 (2)	0 (1)	–
	♂	0 (3)	7 (2)	28 (1)
952059–9	♀	–	0(1)	0 (1)
	♂	0 (1)	–	1 (4)
952400–1	♀	3 (2)	0 (1)	0 (1)
	♂	2 (5)	0 (4)	0 (1)
969023–2	♀	0 (3)	1 (10)	–
	♂	0 (2)	28 (6)	–

* Tests during three seasons confirmed complete sterility.

Table 3. Ploidy level of progeny obtained from 951301-5 after N_2O treatment

Parents		Cross	Number of progeny analyzed	Ploidy level of the progenies	
Female	Male			3x	4x
'Vivaldi'	951301-5	AA × OA	29	22	7
951301-5	'Vivaldi'	OA × AA	12	12	0

chromosomes to the progenies. In order to verify this assumption, as well as to determine if there was any intergenomic recombination, 12 progeny plants were analysed through GISH (Table 4). As was mentioned in Table 3, all were euploids and included eight triploids and four tetraploids. With the exception of one triploid (042923–1) and one tetraploid (042924–6), GISH analysis indeed confirmed that N_2O treated OA hybrids had contributed 12 chromosomes each of O and A genomes. This means, N_2O had most likely induced first division restitution gametes. In the case of two exceptional plants, 042923–1 and 042924–6, though euploids, they possessed variable numbers of O and A chromosomes (Table 4). In these cases, it was obvious, that indeterminate meiotic restitution had occurred. One important result of GISH was the observation of intergenomic recombination (Figures 2a and b) in three of the progeny plants, viz., 042923–1, 042924–1 and 042928–1 (Table 4). There was a clear indication that

Table 4. Genome composition and number of recombinant chromosomes in progenies obtained from the cross of N_2O treated OA hybrid 951301-5 to Asiatic (A) parent

Genotype	Cross	Ploidy	Genome composition O ($^{O}/_{A}$)	A ($^{A}/_{O}$)	Number of recombinant chromosomes
042923–1	AA × OA	3x	10	26(2)	2
042924–1	AA × OA	4x	12(1)	36(1)	2
042924–2	AA × OA	3x	12	24	0
042924–3	AA × OA	4x	12	36	0
042924–4	AA × OA	3x	12	24	0
042924–5	AA × OA	3x	12	24	0
042924–6	AA × OA	4x	13	35	0
042924–7	AA × OA	4x	12	36	0
042924–8	AA × OA	3x	12	24	0
042927–2	OA × AA	3x	12	24	0
042927–4	OA × AA	3x	12	24	0
042928–1	AA × OA	3x	12(1)	24	1

N_2O had induced restitution gametes in these cases but not chromosome doubling in pre-meiotic stages. This was because, premeiotic chromosome doubling would have resulted in tetraploids in which intergenomic recombination was expected to be very rare, if not totally absent.

Discussion

It is evident from this investigation that 'fertility' can be restored in totally sterile interspecific hybrids through N_2O treatments under pressure. Although not all eight genotypes that were treated with N_2O produced positive results, there was *in vitro* germination in five cases (Table 1). At least three of these genotypes could be used either as female or male parents in crosses in order to obtain embryos- and finally progenies. Production of a considerable number of embryos (208) in the case of 951301-5 shows that even highly sterile interspecific hybrids can be used in breeding.

The fact that N_2O treated plants can be successfully used both as male and female parents suggests that functional spores occur in micro- and megasporogenesis. The actual mechanisms of the origin of these functional gametes are not clear from this study because it was not cytologically analysed in treated plants. However, the chromosome constitution of the

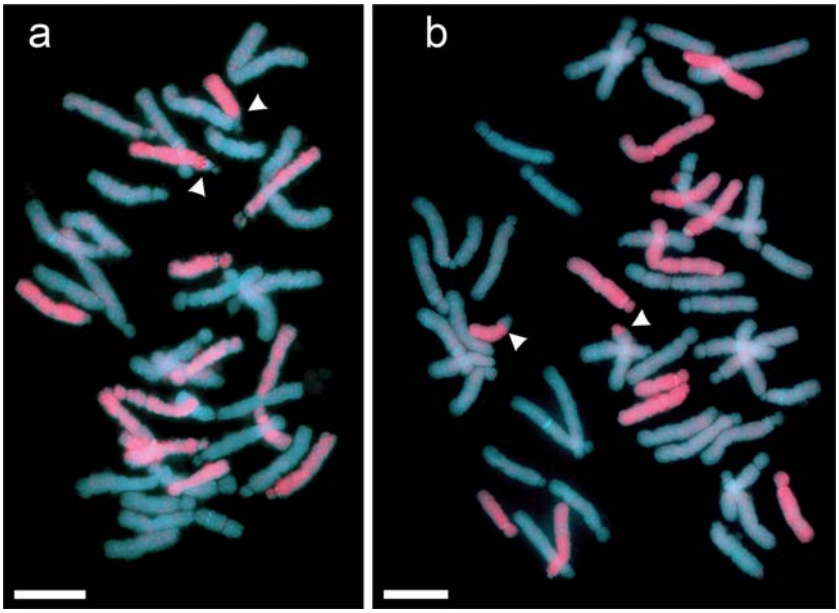

Figure 2. Chromosome complements of the BC_1 progenies from the cross of N_2O treated OA hybrid 951301–5 to Asiatic (A) parent showing a triploid ($2n = 3x = 36$) and a tetraploid ($2n = 4x = 48$) chromosome number. (a) Triploid chromosome complement of 042923-1, showing 10 Oriental and 26 Asiatic chromosomes with two recombinants (arrowheads). (b) Tetraploid chromosome complement of 042924-1, showing 12 Oriental and 36 Asiatic chromosomes with two recombinants (arrowheads). In both cases the biotin-labeled Oriental DNA was detected with the Cy3-streptavidin system (pink fluorescence) and Asiatic chromosomes were counter stained with DAPI (blue fluorescence). *Bar*: 10 μm.

progeny plants (Table 4) suggests that the 2*n* gametes are predominantly of FDR origin. One of the important requirements for the origin of FDR during microsporogenesis in lilies is that the half-bivalents should divide equationally (as in mitosis) before the cytokinesis and cell wall formation occurs in telophase I stage (Lim et al. 2001). If N_2O treatment is assumed to affect meiotic spindle formation during the first meiotic division, as is shown in *Tradescantia* (Montezuma-De-Carvalho 1973), this could lead to mitosis like division of the entire chromosome complement in the treated OA hybrid. Dyads and 2*n* spores that result from such division will indeed be FDR. The presence of recombinant chromosomes does indicate that they are indeed the result of FDR with crossovers. This implies that N_2O had an effect on pollen mother cells undergoing various meiotic stages. This does not exclude, however, that N_2O might have also induced premeiotic doubling. For example, there were two progeny plants that were derived from 2*n* eggs of N_2O treated OA hybrids (042927–2 and 042927–4) but did not possess recombinant chromosomes. In *Lilium*, meiotic stages in anthers initiate when flower buds reach 0.5 to 0.9 cm (Walters, 1976, 1980; Lord & Gould, 1989) and similar stages are present in the embryo sac mother cells (De Boer-De Jeu MJ 1978). However, due to the difference in size of the flower buds at the time of N_2O treatment also premeiotic division stages could have been affected leading to premeiotic doubling. This possibility could not be answered in this study.

One aspect that deserves to be noted is that not all genotypes that were treated with N_2O responded similarly. One reason could be that not all plants used for the N_2O treatments had the same meiotic stages in their anthers. Alternatively, there might be genotypic differences. If it is only due to differences in meiotic stages, then the plants require a more stringent cytological monitoring before treatment. It has been shown that meiosis in anthers of *Lilium longiflorum* has a duration of 50 days (Tylor and McMaster 1954). In such cases, it might be possible to determine the optimal stage for N_2O treatments and maximize the chances of inducing fertility. Although not all genotypes treated with N_2O showed fertility, the ones that became fertile showed that the procedure can be useful. This can open the way for inducing 2*n* gametes in some of the desirable genotypes of OA hybrids.

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