

Cytogenetic analysis of progeny derived from allotriploid inter-specific hybrids of *Lilium*

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The allotriploid cultivar 'Angela North' ($2n = 3x = 36$) possessing one genome of *Lilium lankongense* Franchet and two genomes of Asiatic hybrids (AH), and allotriploid cultivars from LAA group containing one genome of *L. longiflorum* Thunberg and two genomes of AH were backcrossed with diploid ($2n = 2x = 24$) and tetraploid ($2n = 4x = 48$) cultivars of lilies from AH group. Progeny was received only by cultivating isolated embryos on MS media. LAA cultivars for backcrossing were used as female and male, however, cv. 'Angela North' possessed only female fertility. More than half of progeny received after crossing the allotriploid cv. 'Angela North' with diploid or tetraploid AH possessed a diploid set of chromosomes and others were aneuploid with a chromosome number near to diploid or triploid. The number of chromosomes in progenies derived after pollination of LAA cultivars with tetraploid AH ranged from diploid to nearly pentaploid. No viable progeny was received after pollination of LA by diploid and tetraploid AH by LAA. When male fertile LAA cultivars were used to pollinate diploid AH, mainly diploid progeny were received. It shows that viable pollen of allotriploid LAA cultivars possesses a balanced haploid set of chromosomes.

It looks likely that diploid progeny derived after pollination of allotriploid hybrids with tetraploid AH are apomictic. If diploid progeny derived from allotriploids possess recombinant chromosomes and restored fertility, they should be valuable in breeding programs for introgression of traits from *L. lankongense* and *L. longiflorum* in Asiatic hybrids.

Key words: *Lilium lankongense*, *L. longiflorum*, inter-specific hybridization, allotriploid, apomixis

INTRODUCTION

The inter-specific hybridization is an important tool in plant breeding [1, 2]. Inter-specific hybridization in the genus *Lilium* has been conducted to produce novel hybrids that can combine ornamental traits and resistance to virus and fungal diseases from distantly related species. Depending on the origin, hybrid lilies are presently categorized into eight divisions [3]. Cultivars belonging to the same horticultural division are a result of intra-section crosses. For example, widely grown cultivars of lilies belong to Asiatic (AH), Oriental (OH) and Trumpet (TH) hybrids derived from species of *Sinomartagon*, *Archeolyrion* or *Leucolyrion* sections, respectively. Inter-specific hybridization among species of the same section is usually not difficult, and such hybrids possess fertility enough to receive next generations. Such inter-division isolation determines that the genetic potentiality of a genus wasn't explored enough. However, developing methods to overcome pre-fertilization barriers allowed to receive distant inter-specific hybrids that are temporarily categorized as miscellaneous [4, 5]. Recently, as a result of inter-sectional hybridization, new cultivars derived by crossing Trumpet hybrids × Oriental hybrids, *L. longiflorum* × Asiatic hybrids, *L.*

longiflorum × Oriental hybrids, and Oriental hybrids × Asiatic hybrids the lily assortment has been innovated [6, 7].

In *Lilium*, as in most other taxa, wide inter-specific hybrids are in general sterile. Sterility in such hybrids mainly results from irregular chromosome associations between the parental genomes during meiosis [8–10]. The traditional method of restoring fertility in such cases is to double the chromosome set of the F_1 hybrid and produce allopolyploids that might be fertile [10]. Such allopolyploids are appropriately called "permanent hybrids" because their progenies never segregate for parental characters due to the strictly autosyndetic pairing of different genomes in allopolyploids [2]. On the contrary, inter-genomic recombination may occur in sexual polyploids induced through formation of unreduced $2n$ gametes [2, 7, 11, 12].

In the last two decades, *L. longiflorum* as a rapidly developing species possessing complex valuable traits was involved in the breeding of lilies. Some of F_1 hybrids derived by crossing *L. longiflorum* with Asiatic hybrids can show a low fertility via formation of unreduced $2n$ gametes. Such hybrids were backcrossed to diploid AH to produce allotriploid LAA cultivars possessing one genome of *L. longiflorum* and two inherited from AH.

Though species of *Lilium* belonging to the same section are related and usually can be crossed more or less easily producing

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fertile progeny, some of them are genetically isolated [13, 14]. Therefore, such species as *L. papilliferum* Franchet, *L. taliense* Franchet, *L. wardii* F. Stern and *L. lankongense* Franchet, possessing anthocyanes in flower petals from the *Sinomartagon* section, did not make a considerable contribution in producing Asiatic hybrids. However, *L. lankongense* Franchet shows a high resistance to *Botrytis* blight, the most important disease of lilies to which cultivars of AH are very susceptible [3]. The flowers of this species are lilac-rose or rose-red, the trait greatly demanded in AH cultivars. *L. lankongense* was crossed with AH and some species from the *Sinomartagon* section by C. North [15], and inter-specific hybrids were received via embryo culture. The F_1 hybrids showed a certain fertility and after backcrossing with diploid AH cultivars and cultivating isolated embryos produced triploid progeny [3], implying that F_1 inter-specific hybrids produced unreduced $2n$ gametes. Some of such allotriploids and F_1 hybrids were registered as AH cultivars. However, despite resistance to fungal diseases, great vitality and ornamental characteristics, these cultivars are interesting only for amateur gardening but not for commercial horticulture because of their down-facing flowers – the trait inherited from *L. lankongense*.

Recently it has been recognized that allotriploids can be used successfully for introgression in breeding programs of lilies, since the homologous recombinant chromosomes in variable numbers can be transmitted to backcross progenies [16]. In this research, fertility of such allotriploid LAA cultivars and the allotriploid cultivar derived from *L. lankongense* and AH was evaluated, and back-crossing of allotriploids to AH was performed.

MATERIALS AND METHODS

Twenty eight allotetraploid ($2n = 3x = 36$) LAA cultivars derived by crossing *L. longiflorum* with AH (Table 1), one allotriploid cultivar 'Angela North' derived by crossing *L. lankongense* with AH, and AH cultivars or seedlings: diploids ($2n = 2x = 24$) 'Connecticut King', 'Sorbet', 'Silhouette', 'Vivaldi', 'Toscana', 'Nepal', 'Geneva', 0-26-6, 0-21-5, 0-47-4, 0-51-3, 0-50-1 and tetraploids ($2n = 4x = 48$) 'Aristo Tetra', 'Encounter', 'Excalibur' and 'Aberdeen' – were grown in field conditions to perform pollination.

The viability of pollen was estimated by its possibility to germinate *in vitro*. Fresh pollen was germinated at +25 °C during 10 h in a drop of water solution containing 20% of sucrose and 0.02% of boric acid [3].

For pollination, the pollen was brought on a stigma of flowers emasculated before opening. To prevent undetermined pollination, the stigma was isolated with a cap of aluminium foil. The embryos were isolated before opening of swelled pod and cultured *in vitro* in sterile conditions on a solid MS medium supplemented with 3% sucrose [17]. Until germination the embryos were cultured at +25 °C in the dark.

Chromosome counts of parental forms and progenies were made on root tips of plants grown *in vitro* conditions as described by Lim et al., 2000 [10].

RESULTS AND DISCUSSION

Pedigree analysis and chromosome counting confirmed the allotriploid nature of 28 LAA cultivars presented in Table 1 and

of cultivar 'Angela North'. All they possess a chromosome set $2n = 3x = 36$. The LAA cultivars are progenies originated by back-crossing of semi fertile unreduced $2n$ gametes forming F_1 hybrids (*L. longiflorum* × AH) to diploid AH and contain one genome (L) inherited from *L. longiflorum* and two genomes (AA) of AH. The cultivar 'Angela North', registered in 1985, was originated by C. North after back-crossing of diploid F_1 inter-specific hybrid (*L. lankongense* × AH 'Edith Cecilia') [15] with a diploid seedling of AH [(*L. lancifolium* var. *flaviflorum* × 'Destiny') × 'Red Fortune'] [3]. Since this cultivar has 36 chromosomes, it looks likely that it originated after fertilization of an unreduced $2n$ egg of the F_1 inter-specific hybrid by haploid sperm of AH and should have one genome (12 chromosomes) inherited from *L. lankongense* and two genomes (24 chromosomes) of AH.

Despite the allotriploid constitution, 'Angela North' and about half of the LAA cultivars studied can set seeds after pollination by AH (Table 1). We detected that pollination with pollen mixtures produces more seeds in a pod than crossing with separated pollen (data not presented). However, seeds are wrinkled and have a poorly developed endosperm. None of seeds germinated when sown in soil. Because of the failure to germinate in a normal

Table 1. Male and female fertility of allotriploid LAA cultivars

No.	Cultivar	Number of germinated pollen, %	Possibility to set seed after pollination by AH
1	'Connecticut King' (control, fertile AH)	29.2 ± 2.3	+
2	'Close Up'	20.7 ± 2.5	+
3	'Club House'	8.6 ± 1.9	–
4	'Carmel'	7.4 ± 1.5	–
5	'Victoria Falls'	5.8 ± 1.4	–
6	'Take Off'	5.0 ± 1.3	+
7	'Double Joy'	2.5 ± 0.5	+
8	'Aerobic'	2.0 ± 0.9	–
9	'Moneymaker'	2.0 ± 0.6	+
10	'Imperial Love'	2.0 ± 0.7	–
11	'Science Fiction'	1.5 ± 0.7	+
12	'Golden Torch'	1.0 ± 0.6	+
13	'Bestseller'	0	–
14	'Dream'	0	–
15	'Evita'	0	+
16	'Manhatan'	0	+
17	'Mega Star'	0	+
18	'Modern Style'	0	–
19	'Party Time'	0	–
20	'Pride Star'	0	+
21	'Royal Grace'	0	–
22	'Royal Club'	0	–
23	'Rodeo'	0	–
24	'Salmon Queen'	0	+
25	'San Jose'	0	+
26	'San Mateo'	0	+
27	'Spirit'	0	–
28	'Twilight Live'	0	+
29	'Tune Up'	0	+

manner, progenies were received by the embryo rescue method. The embryos were excised with part of endosperm 1–2 weeks before dehiscing of the pod capsule and cultivated in aseptic conditions on MS medium in the dark until germination. The embryos usually started to grow after 10 days and later were grown in the light. Later allotriploids possessing a particular female fertility were used as the mother component to back-cross with AH.

The male fertility of allotriploids was determined as a possibility of pollen to germinate in a water solution containing 20% of sucrose and 0.02% of boric acid [3]. Pollen grains of cultivar 'Angela North' showed a very low viability and only 0.2–0.5% of them germinated. Attempt to perform pollination of AH with 'Angela North' as the male partner failed. The viability of pollen in LAA cultivars ranged from 20.7% to 1% and was lower than in the case of the fertile diploid AH cultivar 'Connecticut King'

($\chi^2 = 19.28$, $P_{HO} < 0.05$) which was used as control (Table 1). Among the 28 LAA cultivars tested, 11 had more or less fertile pollen and were used as the male to perform back-crossing with female AH. Evaluation of fertility in allotriploids has shown that female fertility and male fertility are independent phenomena, since a cultivar possessing male sterility may be female-fertile, or *vice versa*.

Allotriploid ($2n = 3x = 36$) cultivars showing female fertility and those possessing viable pollen grains were used in reciprocal interpollid back-crossing with diploid ($2n = 2x = 24$) and tetraploid ($2n = 4x = 48$) AH. Viable progeny were received only when LAA cultivars were used as male to fertilize diploid AH (AA) or when they were used as female in combination with tetraploid AH cultivars (AAAA) as male (Table 2). The embryos isolated from crossing combinations LAA \times AA and

Table 2. Fertility of interpollid crosses

*Direction of crosses	Number of different combinations	Average number of isolated embryos per one pollinated flower	Viability of embryos %	**Received offspring	Comments
AA \times LAA	16	4.50 \pm 1.12	29.00 \pm 1.89	167 (575)	
LAA \times AA	21	0.03 \pm 0.72	0	0 (21)	Embryos died
AAAA \times LAA	6	0.12 \pm 0.07	0	0 (57)	Embryos died
LAA \times AAAA	6	1.24 \pm 0.62	19.93 \pm 4.95	13 (65)	
'Angela North' \times AA	3	8.50 \pm 2.08	51.50 \pm 5.97	36 (70)	
'Angela North' \times AAAA	2	12.91 \pm 4.06	23.10 \pm 5.23	15 (65)	

* A – genome of Asiatic hybrids (AH), L – genome of *L. longiflorum*.

** The number of isolated embryos is presented in parentheses.

Table 3. Chromosome set in progenies derived from allotriploid hybrids

*Direction of crosses	Number of analyzed progenies	Frequency (%) of:			
		diploids ($2n = 24$)	triploids ($2n = 36$)	tetraploids ($2n = 48$)	aneuploids ($2n = 25-59$)
AA \times LAA	42	95.2 \pm 3.3	0	2.4 \pm 2.4	2.4 \pm 2.4 ($2n = 26$)
					10.0 \pm 9.5 ($2n = 35$)
					10.0 \pm 9.5 ($2n = 37$)
					10.0 \pm 9.5 ($2n = 49$)
					10.0 \pm 9.5 ($2n = 56$)
					14.3 \pm 13.2 ($2n = 27$)
'Angela North' \times AA	7	57.1 \pm 18.7	0	0	14.3 \pm 13.2 ($2n = 26 - 50$)
					14.3 \pm 13.2 ($2n = 29 - 32$)
					12.5 \pm 11.7 ($2n = 25$)
'Angela North' \times AAAA	8	62.5 \pm 17.1	12.5 \pm 11.7	0	12.5 \pm 11.7 ($2n = 29$)

* A – genome of Asiatic hybrids (AH), L – genome of *L. longiflorum*.

AAAA × LAA germinated poorly and soon died. Most of progeny derived from diploid AH × LAA crosses consisted of diploids (Table 3). It confirms that viable pollen of LAA consists mainly of haploid pollen ($n = 12$) with a balanced number of chromosomes. Among 42 offspring only one was aneuploid with two additional chromosomes to a diploid set ($2n = 26$), and one possessed the chromosome number $2n = 48$ characteristic of tetraploids. The latter may be originated from a haploid egg and triploid sperm or from an unreduced egg and diploid sperm. On the contrary, analysis of progeny derived from LAA × tetraploid AH combinations has shown that fertile female gametes formed during meiosis in allotriploids possess a chromosome set ranging from haploid or nearly haploid to nearly triploid, and eggs with an unbalanced set of chromosomes can participate in fertilization and produce the next generation (Table 3). The progeny from LAA × AAAA inter-ploid crosses possessing a diploid set ($2n = 24$) must be interpreted as apomicts. It is known that formation of functional gametes in inter-specific hybrids is connected with recombination events in homologous chromosomes during meiosis [16]. If such recombinant chromosomes were transmitted to diploid progeny received from AA × LAA and LAA × AAAA inter-ploid crosses, such offspring would be valuable in the evaluation of inter-specific hybrids for introgression and breeding at a diploid level.

Since the allotriploid cultivar 'Angela North' shows a female but not a male fertility, it was used as maternal component to perform inter-ploid crossing with diploid and tetraploid AH cultivars (Table 2). Contrary to inter-ploid crosses performed with LAA, allotriploid 'Angela North' produced progeny after pollination by a diploid as well as by a tetraploid male. Although the number of seeds with embryo per seed pod was almost similar for both crossing directions, the viability of embryos in the case of 'Angela North' × AA was approximately twice higher than in combination with tetraploid AH ($\chi^2 = 27.32$, $PHO < 0.05$). The chromosome sets were determined in progenies derived from combination 'Angela North' × AA (mixed pollen of diploid cultivars 'Sorbet', 'Silhouette', 'Vivaldi' and 'Toscana') and from 'Angela North' × AAAA (mixed pollen of tetraploid cultivars 'Aristo Tetra' and 'Aberdeen') (Table 3). More than half of the progenies in both combinations were diploid and possessed 24 chromosomes. The other part of offspring were nearly diploid aneuploids possessing 1–4 additional chromosomes. Two plants derived from crossing with a diploid AH male showed chromosome instability and were mixoploids. Only one offspring originated in crossing with the tetraploid AH was tri-ploid. Unlike in auto triploid, chromosome pairing can be more restricted during meiosis in the case of allotriploids because of differentiation of chromosomes of alien genomes. This can lead to formation of a higher frequency of univalency leading to a more unbalanced meiosis and a higher degree of sterility [18]. Analysis of the chromosome set in the progeny derived from an allotriploid hybrid of *L. lankongense* and diploid AH indicates, that among gametes with an unbalanced number of chromosomes euploids can be formed and egg cells possessing a haploid or nearly haploid number of chromosomes predominantly contribute to the formation of progeny. However, these diploid progenies can be apomictic too. So, chromosome instability detected in two progenies shows that offspring possessing a diploid or nearly

diploid genome constitution may be derived as a result of chromosome loss in the early stage of embryo formation. However, if the haploid egg is fertilized by $2n$ sperm of a tetraploid male, more triploid offspring with 36 chromosomes must be expected. As was detected by us and by Lim et al. [16], in the case of LAA allotriploids, female gametes of LAA with a chromosome set ranging from n to $3n$ may participate in the fertilization and formation of the next generation depending on the ploidy level of male. However, we did not detect pentaploid or nearly pentaploid progenies among the offspring of the allotriploid hybrid of *Lilium lankongense* pollinated by a tetraploid male. It is possible that progenies of the allotriploid hybrid of *Lilium lankongense*, obtained after crossing with the tetraploid AH, originated from an unfertilized egg and are apomictic.

To interpret the differences in fertility depending on cross direction in inter-ploid crosses where LAA are allotriploids the '2.5' rule was proposed [16]. Since the embryo sac of the *Lilium* species is tetrasporic, of the eight-nucleated type and the secondary nucleus in the embryo sac of diploid lilies possesses a set of four chromosomes, the ratio of chromosome number in the embryo and endosperm is 2.5. This ratio should be optimal for embryo development. When a female possesses a lower chromosome set than a male, this ratio is lower, but it dramatically increases if the chromosome set of the female is higher than of the male. However, the possibility to obtain progeny when the allotriploid cultivar 'Angela North' was pollinated by the diploid AH (similar inter-ploid crosses performed with LAA failed) demands another explanation. It may be that a large part of the progeny obtained from 'Angela North' is apomictic diploids.

It has been shown that during meiosis in allotriploids alien chromosomes can cross-over and such recombinant chromosomes are transmitted to the next generation [7, 12, 16]. This allows incorporating allotriploids for introgression of alien genetic information in recipient chromosomes. If diploid progenies of the allotriploid hybrid of *Lilium lankongense* and LAA would possess recombinant chromosomes, they could be expected to be fertile and suitable for breeding at the diploid level.

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ALOTRIPLOIDINIŲ TARPRŪŠINIŲ LELIJŲ HIBRIDŲ PALIKUONIŲ CITOGENETINĖ ANALIZĖ

S a n t r a u k a

Alotriploidinės LAA grupės lelijų veislės, turinčios vieną *Lilium longiflorum* ir du azijinių hibridų (AH) grupės lelijų genomus, taip pat veislė 'Angela North', turinti vieną *L. lankongense* ir du AH genomus, buvo kryžminamos su diploidinėmis ir tetraploidinėmis AH veislėmis. Visų kryžminimų palikuonys buvo gauti auginant izoliuotus gemalus *in vitro* ant MS terpės. Kadangi veislės 'Angela North' žiedadulkės sterlios, kryžminimuose ji buvo naudojama tik kaip motininis komponentas. Daugiau negu pusė šios veislės palikuonių turėjo diploidinį chromosomų rinkinį, kiti buvo aneuploidai. Palikuonių citogenetinė analizė rodo, jog alotriploidinių LAA grupės veislių fertilių žiedadulkės dažniausiai turi subalansuotą haploidinį chromosomų rinkinį. Naudojant LAA grupės veisles kaip motininį komponentą, buvo gauti įvairaus ploidiškumo palikuonys, tarp jų buvo ir apomiktinės kilmės diploidų. Tikėtina, jog diploidiniai alotriploidų palikuonys pasižymės didesniu fertimumu. Kadangi tokie palikuonys galėjo paveldėti ir rekombinantines chromosomas, jie galėtų būti vertingi kuriant naujas lelijų veisles.