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Polyploidization of *Lilium* and *Tulipa*

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SUMMARY

Tulip and lily, which belongs to the genus *Tulipa* and *Lilium,* respectively are the economically most important ornamental crops in The Netherlands. Due to their important economic and ornamental value, breeding plays an important role to create new economically and potentially new cultivars. Polyploidization also plays an important role in the interspecific breeding of lilies and tulips. The reasons for using polyploidy in lily breeding are to obtain larger flowers, stronger stems (especially important for forcing under low light conditions during the winter period) and to restore fertility in F1- interspecific hybrid at the tetraploid level. Diploid gamete selection and artificial chromosome doubling are the two main ways to achieve polyploidization in tulip and lily breeding. There are two aims of this thesis; one is selecting 2*n* pollen producers from F1 hybrids of *T. gesneriana* x *T. fosteriana* tulip hybrids crossing experiment. Furthermore, to test one year old tulip seedlings for ploidy levels of the F1 progenies in order to determine the result of the function of the 2*n* pollen for which the genotypes were previously selected. In this thesis report, two activities have been attempted to achieve Polyploidization in tulip and lily by two different approaches. The second objective was to investigate the efficiency of *in vitro* chromosome doubling by using different concentrations of oryzalin in lily.

For tulip *2n* pollen producer selection experiment, 171 different F1 tulip progenies were tested for the pollen germination rate, then selected the ones that had higher pollen fertility as well as possessed different sizes of pollen grain as determined by measurements. In total 13 of these F1 progenies which had large pollen grain sizes were selected. Based on the selection results, 308 one year old tulip F1 seedlings derived from crosses between diploid cultivars and selected diploid 2n pollen producers (2x X 2x, 202 samples) and triploid cultivars and selected diploid 2n pollen producers (3x X 2x, 106 samples) were tested for the ploidy levels. The result indicated that in the crosses of 2x X 2x, we got the impression as if the 2n pollen is not functional and produced hardly any polyploidy progenies. But in the case of 3x X 2x crosses there was clear evidence that the progenies had resulted from the functioning of *2n* pollen.

Key words: 2*n* gametes, Polyploidization, *Tulipa*, *Lilium*

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Two years ago when I arrived in The Netherlands, I was attracted by the impressive view of the flower fields near De Keukenhof and the outstanding Dutch flower industries. From then on, I realized that flower industry is suitable for my future career, especially after I finish my placement in Testcentrum in Hillegom from February. to June, 2009, my passion for flowers industry was becoming deeper and deeper. Now I am going to graduate and flower industry will be my first option for the future career, especially related to lilies and tulips. It is my great honour to express my gratefulness to everybody who helped me in different ways.

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1 INTRODUCTION

1.1 General aspects

1.1.1 Lilium

The genus *Lilium* belongs to the Liliaceae family which comprises of about 80 species (Comber, 1949) and thousands of cultivars (Leslie, 1982-2005). The common name of *Lilium* is lily and they originated in the Himalayan region and they have extended over the mountain areas in the Northern hemisphere (De Jong, 1974). Based on various morphological and physiological characteristics, the lily species are taxonomically classified into seven sections. They are: Lilium, Martagon, Pseudolirium, Archelirion, Sinomartagon, Leucolirion and Oxypetalum (Comber, 1949).

Wild lily species, which are predominantly diploid (2n=2x=24), within each section are relatively easy to cross and the hybrids are mostly fertile. Most cultivars are interspecific hybrids of species within the sections (especially Leucolirion, Sinomartagon and Archelirion) and represent the most important cultivated groups, namely the **Longiflorum hybrids**, the **Asiatic hybrids** and the **Oriental hybrids** (Van Tuyl *et al.*, 2002).

1.1.2 Tulipa

Tulipa's common name is tulip and it is a perennial plant comprising of about 150 bulbous species with showy flowers, in the family Liliaceae. Tulips originated from southern Europe, north Africa and Asia from Anatolia and Iran in the west to northeast of China. The centre of diversity of the genus is in the Pamir and Hindu Kush Mountains and the steppes of Kazakhstan. The tulip was introduced into the Netherlands from Turkey in the 16th century (Van Tuyl and Van Creij, 2006).

According to the taxonomic classification by Van Raamsdonk *et al*. (1997), the genus is divided into two subgenera: ***Tulipa*** and ***Eriostemones***, and these two subgenera are classified into eight sections:

Subgenus *Tulipa*: *Tulipa, Eichleres, Tulipanum, Kolpakowskianae, Clusianae*

Subgenus *Eriostemones*: *Australes, Saxatiles, Biflores*

Tulipa gesneriana belongs to subgenera *Tulipa*, section *Tulipa*, and is the most important cultivar group. Darwin Hybrids are interspecfic hybrids between *T. generiana* and *T. fosteriana* (Lefeber, 1960). Darwin Hybrid tulips are excellent cultivars because their large flower, sturdy stem and plant size. In the Netherlands, Darwin Hybrids constitute about 8% of the total tulip production area (Marasek *et al*., 2006).

1.2 Polyploidization

Polyploidization plays an important role in the interspecific breeding of lilies and tulips. The reasons for using polyploidy in lily breeding are larger flowers, stronger stems (especially important for forcing under low light conditions during the winter period) and in interspecific hybridization to restore F1- fertility at the tetraploid level (artificial chromosome doubling or mitotic polyploidization) (Van Tuyl *et al*., 1989).

In ornamental plants, interspecific hybridization is the most important source of genetic variation. Most tulip species and cultivars are diploids, but recently more and more triploid and tetraploid cultivars are introduced (Van Tuyl and Van Creij, 2006). In tulip, triploid and tetraploid cultivars generally have larger flowers, stronger stems, sturdy flower segments and withstand transport as cut flowers.

Generally there are two ways for polyploidization which can be categorised into mitotic polyploidization and meiotic polyploidization (Lim and Van Tuyl, 2006). In the case of mitotic polyploidization chromosomes are doubled by treating vegetative tissues which spindle inhibitors like colchicine (Blakeslee and Avery, 1937) or oryzalin (Van Tuyl *et al*., 1992). However due to the preferential pairing between homologous chromosomes at metaphase I of the meiosis in induced allopolyploids, there is a lack of

homoeoloeogous recombination between parental genomes. Therefore in case of mitotic polyploidization the introgression of characters in the next generation is limited.

In the case of meiotic polyploidization (sexual polyploidization) 2*n* gametes which occur occasionally in interspecific hybrids are directly formed in diploid plants. This leads to homoeologous recombination between the parental chromosomes. This obviously facilitates intergenomic recombination in the progenies. In addition, 2*n* gametes can transmit broad heterozygosity to the polyploid offspring (Lim *et al*., 2000). Because 2*n* gametes can overcome F1 sterility in interspecific hybrids, induce intergeneric recombination as well as promote hybrid vigour, these are important in breeding (Van Tuyl *et al*., 1989).

1.3 Research Objectives

i. Selecting (unreduced gamete) 2*n* pollen producing F1 hybrids (2*n* pollen producers) of *T. gesneriana* x *T. fosteriana* tulips that can be used for crossing experiment.

ii. Investigating the efficiency of *in vitro* chromosome doubling by using different concentrations of oryzalin in lily.

1.4 Research questions

1.4.1 How to define which tulip F1 hybrid genotypes of *T. gesneriana* × *T. fosteriana* will produces 2*n*-pollen that can be used for polyploidization?

1.4.1.1 How to define pollen fertility?

1.4.1.2 How to identify 2*n* pollen in tulip?

1.4.1.3 Does the *2n* pollen size differ in different genotypes?

1.4.1.4 What polyploid levels of progenies are produced when 2n pollen producing diploid genotypes are used in 2x X 2x (diploid mother crosses diploid father) and 3x X 2x (triploid mother crosses diploid father) crosses?

1.4.2 Is oryzalin an efficient compound for *in vitro* chromosome doubling of certain lily genotypes?

1.4.2.1 Which oryzalin concentration (0.003% and 0.001%) results in higher number of polyploid plants?

1.4.2.2 Do different genotypes give different polyploidy results?

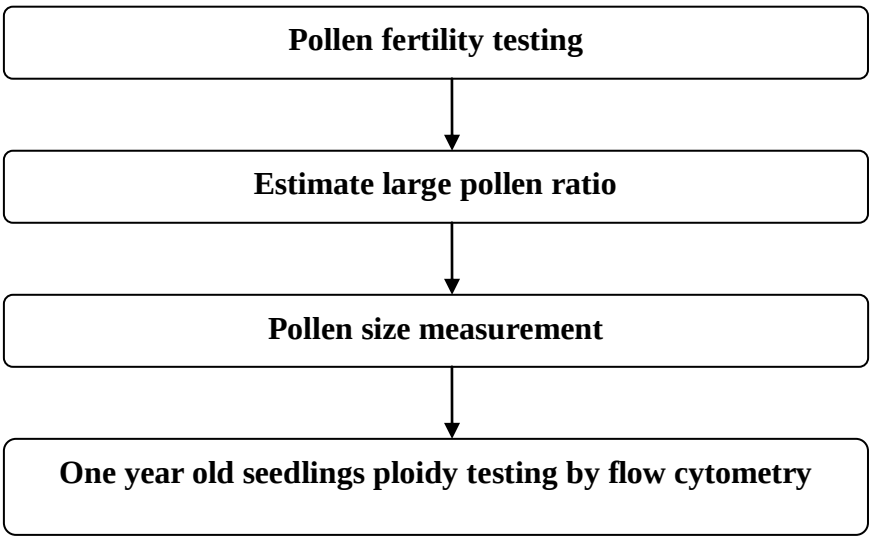
2 RESEARCH METHODS AND PROCEDURES

2.1 Pollen analysis

Material

The experimental material consists of 171 different F1 genotypes from the interspecific crosses between *T. gesneriana* x *T. fosteriana*, these flower bulbs of which were grown at PRI.

Methods



Step 1: Pollen fertility testing

Tulip anthers were emasculated by clean forceps when they started blooming in the glasshouse. Fresh pollen grains were stored in the small Petri dishes and dried in the desiccators in the glasshouse (the desiccators should put under shadow in order to avoid sunlight irradiating on the pollen grains directly). When pollen grains were dry (normally it will take 18 hours), pollen grains were cultured in germination medium (100g/L sucrose, 20mg/L Boric Acid, 200mg/L Calcium Nitrate + 5g/L Agar, pH~5.8) and stored the Petri dishes in the same desiccators in the glasshouse (no additional condition needed, just stay in the glasshouse condition). After one day, germinated pollen tubes were

observed by using light microscope and the pollen fertility was recorded by counting how many pollen gives tubes in total tested pollen because only fertile pollen are able to produce tubes. Finally the genotypes which had acceptable levels of fertility (≥5%) were selected and those genotypes were used for conducting 2*n* (large) pollen test.

Step 2: Estimation of the frequency of large pollen

Fresh pollen grains from genotypes which had relatively higher fertility (≥ 5%, selected from step 1) were brushed on the solid medium (20g/L Daishin Agar and water), then observed by using light microscope. Generally 2*n* pollen grain had twice the DNA content as compared to haploid pollen grains, which means 2*n* pollen grains diameter is approximately twice as large as haploid pollen grain (Figure 1). The bigger pollen ratio of each genotype was approximately estimated by counting the number of large pollen grains out of total 50 pollen grains of each genotype, and selected genotypes which had relatively higher proportion of large pollen (≥ 10%) for the next steps.

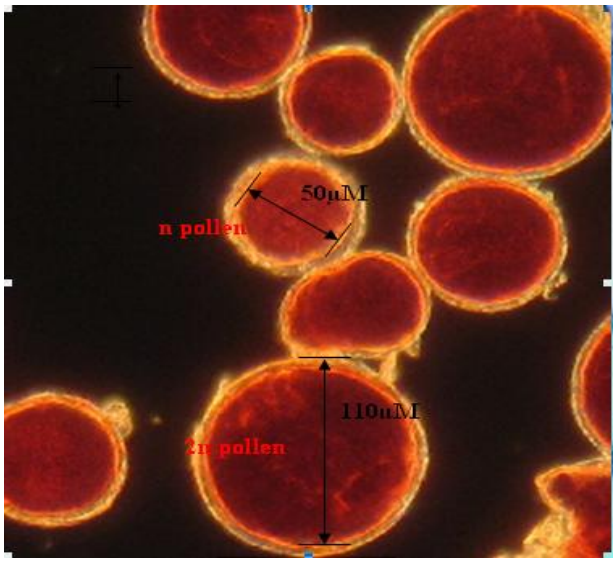


Figure 1. A microscopic view of tulip F1 hybrid pollen sample (date: 19-03-2010, by Shurui. Zhang)

Step 3: Pollen size measuring

Selected genotype's pollen (use fresh pollen again) from previous steps were stained by Lactophenol acid fuchsin (20 g phenol, 20 ml Lactic acid (90%), 40

ml Glycerine (98%), 20 ml water, 80 mg Acid fuchsin). The solution was made in a brown bottle and stirred thoroughly.). After staining, photos of stained pollen were made by light microscope with 10 x 1 magnification. Pollen grain sizes were measured by computer software MicroMeasure, and record data into Microsoft Excel. Finally the genotypes which had 2*n* pollen grain sizes were selected for the crossing experiments (see 2.2).

Step 4: Ploidy testing in one year old seedlings of tulips through flow cytometry

The flow cytometry analysis was done by the breeding support laboratory IRIBOV (a Dutch company which is specialized for testing polyploidy of plants), the Netherlands. Flow cytometry is a technique for counting and examining microscopic particles, such as cells, nuclei and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus (www.iribov.nl/iribov1/analyse_lab.html).

Seedlings derived from crosses either between diploid *T. gesneriana* (2x) and diploid GF (*T. gesneriana* x *T. fosteriana*) (2x) or triploid *T. gesneriana* (3x) and diploid GF (2x) were selected, All the diploid male parents produced large pollen grains based on the results described above. Tips of each seedling were cut by scissors. In total 308 F1 seedlings were tested, and among these 202 were from cross between 2x X 2x, and 106 of them were from 3x X 2x crosses.

2.2 Crossing experiments

After pollen grain size analysis, the selected *2n* pollen producers were used for the crossing experiment in the tunnel and also on the tulip testing field. Crosses were made between 15th and 29th of April, 2010. In total 2449 tulip plants were crossed, mostly the popular cultivars (*T. gesneriana*) were used as mothers and the selected 2*n* producers were as fathers. The crossing experiment result will be available next year.

2.3 Chromosome doubling

Material

Lily bulb scales were obtained from PRI. Three different genotypes (OT, TO and TT) of a total of seven plant groups were used as Table 1. Due to the lack of materials, there was no control treatment for group numbers 61099, 61098, 61097 and 61039, because control treatment cannot give polyploid results.

Table 1. Lily bulb scale chromosome doubling experiment details.

Genotype	Number	Experiment Date	Number of starting scales Concentrations		
			0.001%	0.003%	control
TT	062433	2010/2/18	36	36	43
TT	061099	2010/2/23	21	24	0
TT	061098	2010/2/23	35	37	0
TT	061097	2010/2/24	40	34	0
OT	061039	2010/2/24	38	34	0
TO	082171	2010/2/25	10	10	5
OT	082154	2010/2/25	14	15	5

Methods

Lily bulb scales were split and washed with demi water, the rotten scales were thrown away. **The following steps were done in the sterile area.** Lily bulb scales were cleaned again by 2% hypochlorite (half Liter) for 20 minutes. After sterilizing the scales, hypochlorite was washed away by sterile water 3 times (1 minute, 5 minutes and 10 minutes). After washing, the bottom of each scale was cut because hypochlorite can destroy the cells from wounding areas. If the scales are too big, there were cut into 2 or even 3 pieces in the middle of the scale, and then the scales were dried in flow cabinet for 5 minutes. Oryzalin solution was made before and there were two different oryzalin concentrations and one control (only for 3 groups) were used in the experiment, 0.001% and 0.003% (0.001% = 75 µl/150ml

sterile water, 0.003% = 225 µl/150ml sterile water), always shaking the beaker while diluting the oryzalin, otherwise crystals will be formed. When scales were dry, they were placed in the oryzalin working solution for 3 hours. After 3 hours, scales were washed again with sterile water 3 times (1 minute, 5 minutes and 10 minutes), and the scales were placed into Petri dishes and dried for 5 minutes. Scales were transfered into lily propagation media (Morashige and Skoog salt with vitamins 2.2g/l, sucrose 50g/l and Gelrite 4g/l, pH ~ 5.8), one scale per plastic tube in order to avoid infections from other scales, then the tubes were labeled. Finally the tubes were stored in dark at 25 ℃ in the environment room of Plant Breeding Group in PRI.

The tubes were checked at least once a week, and the ones that were infected were thrown away. After several weeks, young bulbs were formed in the bottom of scales and then the number of remaining scales for each genotype and treatment was record. When the new bulbs were big enough, they were transplanted to new lily propagation medias (same as before) to make sure that they had enough space to grow. Before transplanting, a small piece of the each young bulb (can be a piece of root, seedling or scale) was taken for the flow cytometry testing (as per Van Tuyl *et al*., 1992).

3 RESULTS & DISCUSSION

3.1Unreduced (*2n*) pollen analysis in tulip

3.1.1 Pollen fertility test based on germination

There were 171 different genotypes of tulip growing under greenhouse conditions and tulips started blooming on 1st March. In total, pollen fertility of 684 individual F1 hybrid genotypes of *T. gesneriana* and *T. fosteriana* were tested, of which 280 of them showed germination rate higher than 1%, and 226 of individuals showed germination rate higher than 5%.

Appendix I shows the pollen fertility results. From the results we can see that there were 9 F1 hybrids which were derived from crosses between KeesNelis and Cantata having higher pollen fertilities, 45 F1 progenies derived from crosses between cultivars and Darwin Hybrids also having higher pollen fertilities (because reasons like virus and disease, some plants could not bloom so that there was no data recorded).

3.1.2 2n pollen identification

The estimation of *2n* pollen was done after pollen fertility testing. Pollen samples from 54 F1 hybrids（selected in 3.1.1）with relatively higher pollen fertility (higher than 5%) and one cultivar (090004) as control were analysed.

After pollen size measurement, there were 17 F1 hybrids which were able to produce larger pollen grains as compared with the diploid control cultivar's pollen size. Seven of 17 F1 genotypes were selected for coming flow cytometry testing (for the other genotypes, there were no F1 seedlings available from last year). Table 2 shows the range of pollen size of these 17 hybrids and one control cultivar

(090004 Kees Nelis). Highlighted genotypes 20168-3, 10170-7, 20190-4, S-20168-2, S-20253-1, 20230-9 and 20241-2 were the selected ones for flow cytometry testing.

Table 2.Pollen sizes of 17 selected hybrids' pollen size + 1 controls.

Genotype	Average Pollen length (µm)	Minimum pollen length (µm)	Maximum pollen length (µm)
20251-1	47.49	35.82	79.99
090004	55.19	39.64	69.78
89191-24	61.93	37.36	98.83
20251-2	63.57	48.91	120.45
20231-1	69.78	41.75	110.90
89191-96	73.36	54.90	110.57
S-20253-1	76.20	40.97	128.60
20259-12	76.36	38.06	126.98
S-20186-2	76.74	48.82	137.25
20241-2	78.35	51.04	119.91
89191-111	79.92	53.63	113.67
20170-4	83.21	36.33	126.29
20185-4	87.10	67.52	110.60
20168-3	87.31	60.57	129.11
20190-4	88.93	65.68	116.99
20230-9	89.03	69.69	118.25
20232-2	89.16	58.89	109.85
20170-7	89.78	54.24	113.87

In general, *2n* pollen is 1.7 times bigger than *n* pollen (Sato., *et al*, 2010), in this experiment, it was considered the pollen grain which had 1.7 times bigger size than the control cultivar's average pollen grain size as *2n* pollen.

From Table 3 we can see the 7 selected F1 genotypes' *2n* pollen percentages. According to the result, genotype 20168-3 and 20190-4 both has highest 2*n* pollen percentage compare to the others, respectively 82.78% and 86.25%.

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Table 3. 2*n* pollen percentage of selected 7 F1 hybrid.

F1 Hybrids	2*n* pollen percentage
20168-3	82.78
20170-4	47.14
20190-4	86.25
S-20186-2	31.30
S-20253-1	18.13
20230-9	8.47
20241-2	25.40

From Figure 2 to Figure 6, it can be seen that the pollen grain size distributions in percentages in the 7 certain selected F1 genotypes. At least 100 pollen grains' sizes were measured in order to get realiable data. Figure 2 shows the distributions of the certain 3 cultivars which only have one peak point in a high size level (85µm -95µm), they might be the cultivars which produce large amount of 2*n* pollen.

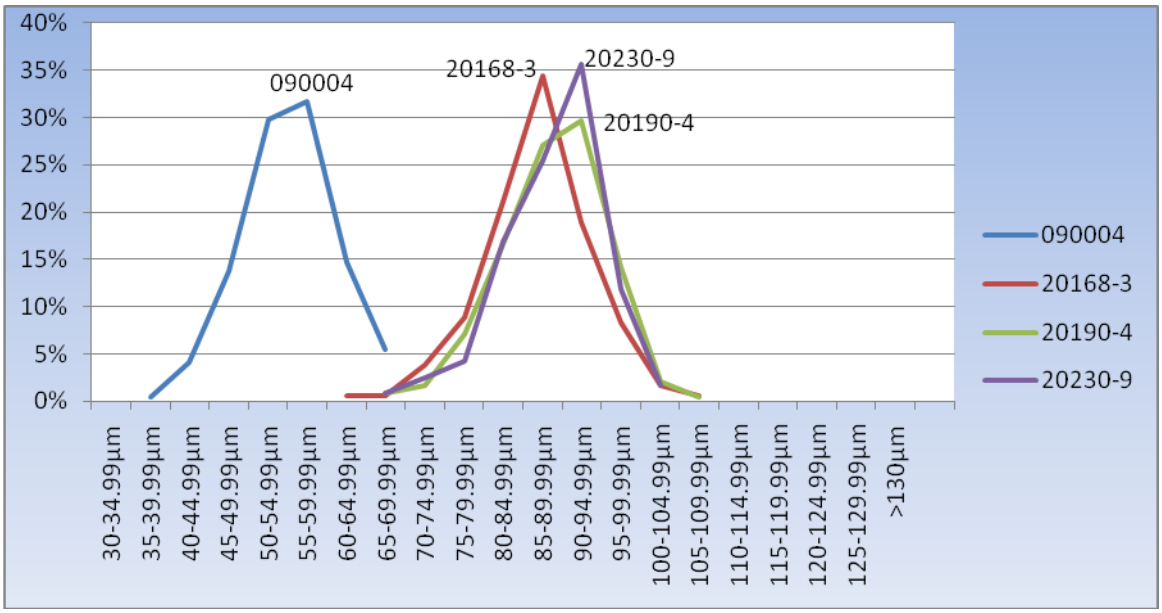

Figure 2 Distributions of pollen grain sizes in percentage of 20168-3, 20230-9, 20190-4 and control cultivar 090004

From figure 2 to figure 6, these certain cultivars all have 2 peak points, especially for S-20168-2, S-20253-1 and 20241-2. The pollen grain size near the first peak point (in the lower size distribution) may be considered as *n* pollen and pollen grain size near the second peak point (in the higher size distribution) may be considered as 2*n* pollen. The ratio of $n/2n \approx 3/1$.

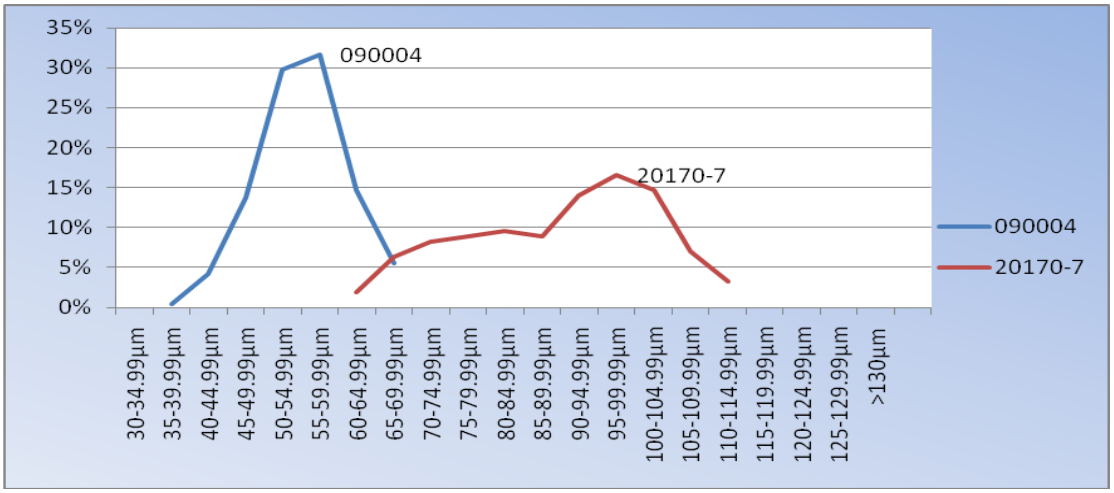

Figure 3 Distributions of pollen grain sizes in percentage 20170-7 and control 090004

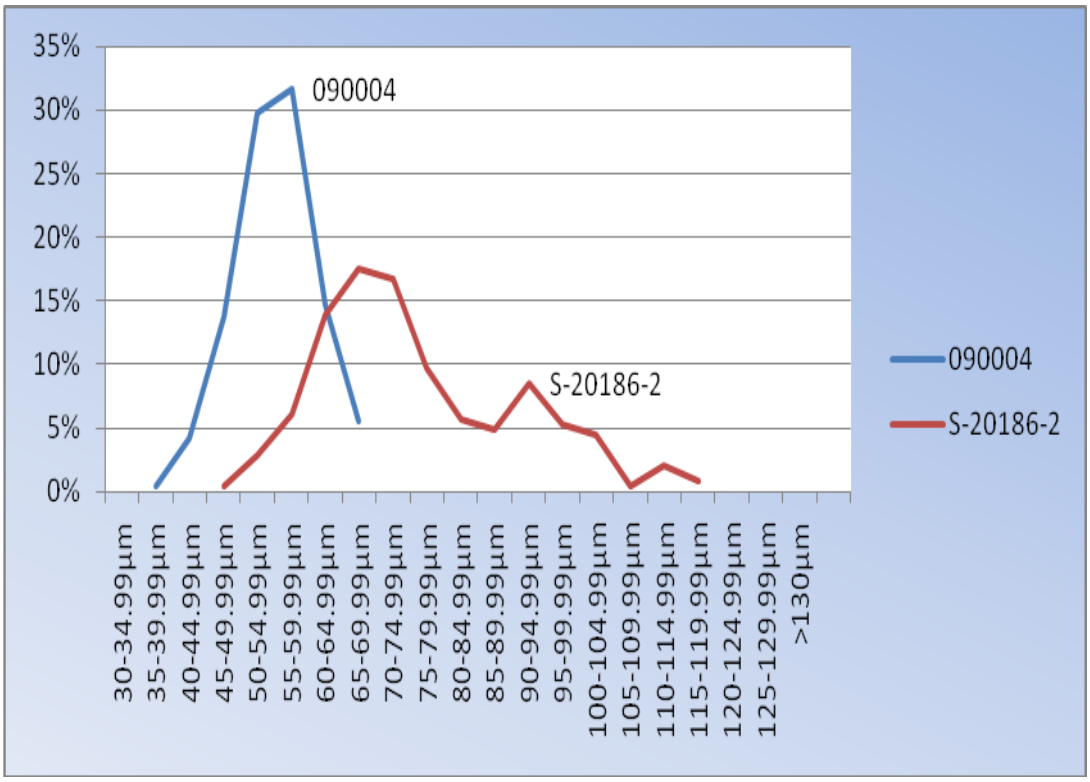

Figure 4 Distributions of pollen grain sizes in percentage S-20186-2 and control 090004

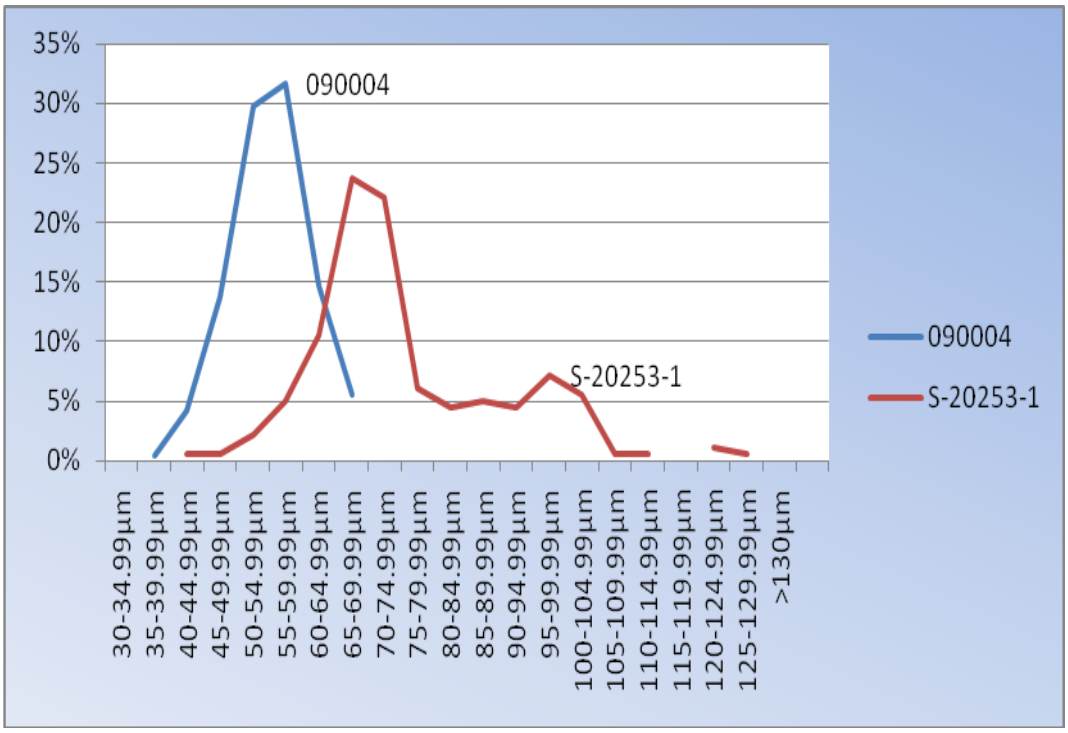

Figure 5 Distributions of pollen grain sizes in percentage S-20253-1 and control 090004

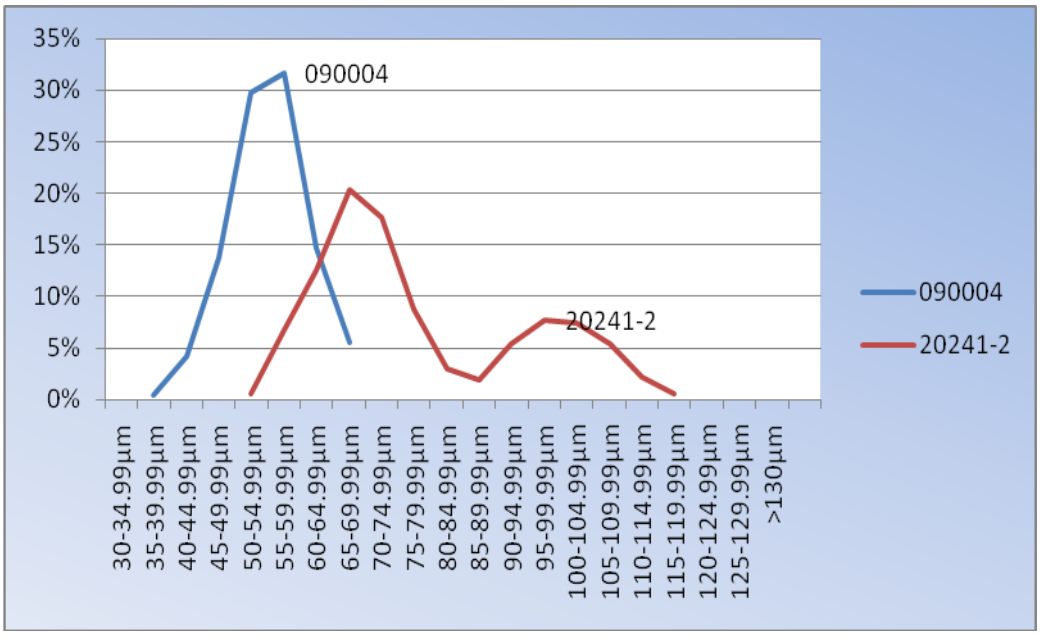

Figure 6 Distributions of pollen grain sizes in percentage 20241-2 and control 090004

3.1.3 Flow cytometry testing results

Flow cytometry testing was done by breeding support company, IRIBOV. In total 308 different one year F1 seedling genotypes have been tested, of which 202 of them were crossed between diploid *T.*

gesneriana and diploid GF (*T. gesneriana X T. fosteriana*) 2*n* pollen producers (2x X 2x) (see Appendix II), 106 of them were crossed between triploid *T. gesneriana* and diploid GF 2*n* pollen producers (3x X 2x) (see Appendix III) and all the fathers were the 7 selected *2n* pollen producers. As we can see from Table 4, with crosses 2x X 2x, 193 of them were diploid according to the flow cytometry testing result, only 9 of them were triploid. But for crosses 3x X 2x, there were 81 of them were tetraploid and 25 of them were even pentaploid.

Table 4. Ploidy levels of tulip seedlings drived from crosses of 2x and 3x mothers with 2*n* pollen producers.

Cross	No. of progeny analysed	Ploidy levels of the progeny			
		2X	3X	4X	5X
2x X 2x	202	193	9	0	0
3x X 2x	106	0	0	81	25

As Appendix II shows, most F1 progenies which crossed between diploid (2x) mothers and diploid (2x) fathers (2*n* pollen producers) were diploid, but we also found 9 triploid seedlings, which means the 2*n* pollen grains were functional to be able to produce triploid progenies, but less efficient, because the amount of triploid progenies were rather low, approximately 5%. However, according to Appendix III, the 2*n* pollen producer seems more efficient in 3x X 2x than 2x X 2x. There were 81 tetraploid and 25 pentaploid progenies found (approximately 25%) and the ratio tetraploid/pentaploid ≈ 3/1, it was approximate the haploid/diploid pollen ratio in 2*n* pollen producers 20241-2, S-20186-2 and S-20253-1 (Figure4, 5, 6 and Appendix III). This is confirmed by S-20253-1 in the 3x X 2x. Also pentaploid lily was found about 10 years ago in 3x X 2x by Lim *et al.* (2002). For tulips this new phenomenon can be seen as a milestone in the tulip breeding history.

3.1.4 Discussion of 2n pollen experiment

After the flow cytometry testing, the result showed that *2n* pollen producers were less effective in the crossings between diploid *T. gesneriana* and diploid GF 2*n* producers (2x X 2x) comparing with crosses between triploid *T. gesneriana* and diploid GF 2*n* producer (3x X 2x) crosses. In order to find out the reasons, advices were given by my supervisor Jaap van Tuyl and Mr. Ramanna Munikote, the expert of gamete field.

In tulips, the embryo sac originates through tetrasporic 8-nucleate system, which means that all the four nuclei that are formed at the end of meiosis are included in the formation of embryo sac. Therefore, the secondary (or endosperm) nucleus and the egg cell (or megaspore) will possess ploidy levels of 4x and x chromosomes respectively. When there is normal fertilization, a haploid nucleus of the sperm cell will fertilize the egg cell and another haploid nucleus, the endosperm nucleus (i.e. double fertilization). As a result of this, an embryo with a 2x nucleus and an endosperm with a 5x nucleus is formed normally. In other words, the ratio of ploidy levels between the embryo: endosperm correspond to 1:2.5. If there is any deviation from this embryo/endosperm ratio occurs, then such embryos may be at a disadvantage in competition with normal embryos. For example, if a 2*n* pollen if functional, then we should expect an embryo with 3x chromosomes and an endosperm with 6x chromosomes. This will give a embryo/endosperm ratio of 1:2 and this may not compete effectively with normally formed embryos when present together in the same ovule. It might explained the very few number of triploids in the tulip progenies of 2x X 2x crosses (Table 4 and Appendix II).

On the other hand, in 3x X 2x crosses, the products of fertilization are expected to possess different embryo/endosperm ratios for the following reasons: in 3x plants the ploidy of endosperm nucleus in

always 6x and that of the egg nucleus can vary anywhere between 12 to 36 chromosomes (with a range of aneuploids). Supposing the egg cell possesses 3x (and the endosperm nucleus being always 6x) and a haploid nucleus has fertilized, then we should expect an embryo with 4x and an endosperm with 7x chromosomes. This gives an embryo/endosperm ration of 1:1.7. Similarly, if 2*n* pollen has been functional in 3x X 2x cross, then we should expect an embryo with 5x and an endosperm with 8x chromosomes. This gives embryo/endosperm ratio of 1:1.6. It may be observed that both of these ratios are abnormal as compared to the ratio observed for normal diploid (1:2.5). Therefore, in 3x X 2x crosses the competition between different products of fertilization may not be as great as in 2x X 2x (non-reduced pollen) crosses. It was proved by Carputo *et al.,* (2002) in Solanums and Ehlenfeldt *et al.,* (1984) in potatoes. Comparing to their results, this might be the reason for the occurrence of both 4x and 5x tulip progenies in 3x X 2x crosses in spite of having an abnormal embryo/ endosperm ratio (Table 4 and Appendix III)

3.2 **Lily bulb scale chromosome doubling**

After different concentrations of Oryzalin treatments, young lily bulbs were formed in the wound area of the scales. Due to the artificial problem like talking while working in the sterile flow cabinet, working with dirty hands… the fungi and bacteria carried in the bulb scales themselves, the infected samples were thrown away. Table 5 shows the starting bulb scales number, remaining bulb scales number and the amount of young bulbs. As we can see, most scales were thrown away because the bacteria and fungi infections, especially number 061039 was completely fail in the end of the experiment, there was nothing left.

Table 5. Lily bulb scale chromosome doubling by different oryzalin concentrations and results.

Genotype	No.	Experiment Date	Checking date	No.of starting scales			No. of remaining scales			No. of young bulbs		
				0.001%	0.003%	control	0.001%	0.003%	control	0.001%	0.003%	control
TT	062433	2010/2/18	2010/5/23	36	36	43	9	8	3	9	4	6
TT	061099	2010/2/23	2010/5/23	21	24	0	1	5	0	2	3	0
TT	061098	2010/2/23	2010/5/23	35	37	0	13	12	0	10	27	0
TT	061097	2010/2/24	2010/5/23	40	34	0	18	13	0	15	3	0
OT	061039	2010/2/24	2010/5/23	38	34	0	fail	fail	fail	fail	fail	fail
TO	082171	2010/2/25	2010/5/23	10	10	5	5	5	0	1	1	4
OT	082154	2010/2/25	2010/5/23	14	15	5	6	8	3	4	6	0

There were 34 pieces (including one control) of lily bulb tissues which obtained from 6 genotypes have been tested. Table 6 shows the detail of the flow cytometry testing results (See Appendix IV for the original testing result). According to the result, for 0.001% Oryzalin treatment, 4 of 18 samples were tetraploid, polyploidy percentage was around 22%; for 0.003% Oryzalin treatment, 4 and 1 of 15 were tetraploid and octaploid, respectively, polyploidy percentage was around 33%. But due to the lack of testing samples, the result cannot give the convictive answer to which oryzalin concentration gives higher number of polyploid plants.

According to the result, almost every genotype gives tetraploid result except 061039 (completely fail because the infection) and 082171, but for 082171, there are still testing materials (young bulbs that are not big enough for testing) left. But by using this method, the first tetraploid *Lilium. henryi* X *Lilium. candidum* obtained from the oryzalin treatment came into flower and showed a fully restored fertility. This breeding material was released to the lily breeding firms in 1990 (Van tuyl., *et al* 1990). In cell cultures of potato and tobacco (Verhoeven *et al*., 1991; Sree Ramulu *et al*., 1990) and in maize callus (Wan *et al*., 1991) oryzalin proved also to be the most efficient chromosome doubling agent as compared to APM (Tokunol M, a phosphoric amide herbicide) and colchicines.

Table 6. Flow cytometry testing result for lily bulb chromosome doubling. (testing date: 2010-06-17)

Number	Genotype	Amount	Oryzalin concentration	Ploidy		
				2x	4x	8x
062433	TT	1	Control	1		
062433	TT	3	0.001%	3		
		3	0.003%	1	2	
082154	OT	3	0.001%	1	2	
		2	0.003%	2		
061097	TT	6	0.001%	5	1	
		1	0.003%		1	
061098	TT	5	0.001%	4	1	
		4	0.003%	4		
061099	TT	0	0.001%			
		4	0.003%	2	1	1
082171	TO	1	0.001%	1		
		1	0.003%	1		
Total		18	0.001%	14	4	
		15	0.003%	10	4	1

4 CONCLUSIONS AND RECOMMENDATIONS

The use of 2*n* gametes and chromosome doubling by oryzalin are both effective ways in plant polyploidization.

Unreduced (2*n*) gamete were successfully used in tulip 3x X 2x crosses and the pentaploid tulips were produced for the first time, 3x and 4x tulip should be used as mother for crossing in the future crosses.

One year seedlings can be tested for ploidy level.

More lily bulb samples should be tested in order to find out which oryzalin concentration will be ideal for chromosome doubling.

One year tulip seedlings ploidy level should be tested next year, especially the ones which crossed by 2*n* pollen producers this year.

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APPENDIX I: Pollen germination testing results.

Name	number	Number of bulbs	species	Germination rate %				
				WK 1	WK 2	WK 3	WK 4	WK 5
KeesNelis x Cantata	89191-16	5	Darwin Hybrid	5	5	20	5	15
KeesNelis x Cantata	89191-24	5	Darwin Hybrid	0	20	30	2	10
KeesNelis x Cantata	89191-41	5	Darwin Hybrid	0	15	2	5	15
KeesNelis x Cantata	89191-58	5	Darwin Hybrid	0	5		5	15
KeesNelis x Cantata	89191-67	5	Darwin Hybrid	40		20	40	30
KeesNelis x Cantata	89191-89	5	Darwin Hybrid	0	15	5	5	5
KeesNelis x Cantata	89191-90	5	Darwin Hybrid	25	5	0	10	30
KeesNelis x Cantata	89191-96	5	Darwin Hybrid	10	10	40	20	
KeesNelis x Cantata	89191-111	5	Darwin Hybrid	10	1	10	5	
Friendship	38002	5	*T. gesneriana*	2		30	20	40
Sachi	38003	5	*T. gesneriana*	2	20	50	15	35
P9	38008	5		20		40	50	
Jan ReusxRecreado	38009	5	*T. gesneriana*	60	30	50	40	
Strong Gold	33102	4	*T. gesneriana*		40	40	40	
White Marvel	33103	5	*T. gesneriana*	10	40	50	40	30
Black Diamond	1416	5	*T. gesneriana*		60	50	50	
ReinierPaping	1424	5	*T. gesneriana*		50	40	40	
Viking	1468	5			50	60	1	25
Monte Carlo	90001	50	*T. gesneriana*	50	50	60	50	40
Christmas Dream	90002	50	*T. gesneriana*	5	60	50	50	50
Bellona	90003	50	*T. gesneriana*	2	40	20	40	50
KeesNelis	90004	50	*T. gesneriana*	0	10	50	50	50
Cantate	90005	50	*T. fosteriana*	80	50	50	50	60
Ile de France	90006	50	*T. gesneriana*	10	50	30	50	50
Bellona x (102 Juan x Cantata)	20160-1	5	Darwin Hybrid	1	15	5	5	1
Bellona x (102 Juan x Cantata)	20160-4	5	Darwin Hybrid	30	5	5	10	5

Bellona x (102 Juan x Cantata)	20160-5	5	Darwin Hybrid	1	3	5	1	5
Bellona x (106 Juan x Cantata)	20164-1	5	Darwin Hybrid	0	15	5	15	5
Bellona x (106 Juan x Cantata)	20164-2	5	Darwin Hybrid	10	5	5	30	5
Bellona x (106 Juan x Cantata)	20164-4	2	Darwin Hybrid			30		
Bellona x (107 Juan x Cantata)	20165-2	5	Darwin Hybrid	30	5	10	10	
Bellona x (108 Juan x Cantata)	20166-1	2	Darwin Hybrid					10
Bellona x (110 Juan x Cantata)	20168-3	2	Darwin Hybrid			30		25
Bellona x (112 Juan x Cantata)	20170-4	2	Darwin Hybrid			25		
Bellona x (112 Juan x Cantata)	20170-7	5	Darwin Hybrid	1	40	25	20	30
Bellona x (113 Juan x Cantata)	20171-8	5	Darwin Hybrid	25	10	20	15	5
Bellona x (118 Cantata x Juan)	20176-3	5	Darwin Hybrid	2	40	35	5	
Bellona x (121 Cantata x Juan)	20179-1	5	Darwin Hybrid	5	5	20	25	20
Bellona x (122 Cantata x Juan)	20180-3	2	Darwin Hybrid			20		20
Bellona x (135 Cantata x Mad. Lef.)	20185-2	5	Darwin Hybrid	40	5	30	30	20
Bellona x (135 Cantata x Mad. Lef.)	20185-4	5	Darwin Hybrid	1	30	20	20	30
Bellona x (137 Cantata x Mad. Lef.)	20187-12	5	Darwin Hybrid	5	5	5	10	15
Bellona x (143 Princeps x Mad. Lef.)	20190-4	2	Darwin Hybrid		30	30		20
Bellona x (148 Cantata x Princeps)	20193-5	2	Darwin Hybrid			30		30
Bellona x (148 Cantata x Princeps)	20193-7	5	Darwin Hybrid	2	5	10	10	20
Ile de France x (121 Cantata x Juan)	20214-2	2	Darwin Hybrid			20		
Ile de France x (137 Cantata x Mad. Lef.)	20221-3	2	Darwin Hybrid			5		
Ile de France x (138 Cantata x Mad. Lef.)	20222-4	2	Darwin Hybrid			5		
Ile de France x (155 Princeps x Cantata)	20230-9	5	Darwin Hybrid	5	40	40	30	15
Generaal de Wet x (102 Juan x Cantata)	20231-1	5	Darwin Hybrid	30		25	40	30
Generaal de Wet x (102 Juan x Cantata)	20231-8	2	Darwin Hybrid			5		2
Generaal de Wet x (104 Juan x Cantata)	20232-2	5	Darwin Hybrid	5	10	40	50	
Generaal de Wet x (104 Juan x Cantata)	20233-1	2	Darwin Hybrid			20		10
Generaal de Wet x (104 Juan x Cantata)	20233-10	2	Darwin Hybrid			40		
Generaal de Wet x (104 Juan x Cantata)	20233-7	5	Darwin Hybrid	2	5	50	20	5
Pax x (102 Juan x Cantata)	20241-2	5	Darwin Hybrid	2	60	50	30	30
Pax x (104 Juan x Cantata)	20242-1	5	Darwin Hybrid	0	50	1	1	25
Pax x (135 Cantata x Mad. Lef.)	20251-1	5	Darwin Hybrid	5	30	5	3	1
Pax x (135 Cantata x Mad. Lef.)	20251-2	5	Darwin Hybrid	5	5	5	2	5
Pax x (147 Cantata x Princeps)	20255-2	5	Darwin Hybrid	0	15	10	5	10
Pax x (149 Princeps x Cantata)	20256-2	2	Darwin Hybrid	0		10		

Pax x (149 Princeps x Cantata)	20256-3	2	Darwin Hybrid			20		10
Pax x (154 Princeps x Cantata)	20258-1	5	Darwin Hybrid	2	10	20	15	10
Pax x (155 Princeps x Cantata)	20259-1	5	Darwin Hybrid	10	30	50	40	
Pax x (155 Princeps x Cantata)	20259-12	5	Darwin Hybrid	5	1	20	10	15
Pax x (155 Princeps x Cantata)	20259-13	5	Darwin Hybrid	1	10	1	3	15
Pax x (155 Princeps x Cantata)	20259-23	5	Darwin Hybrid		60		20	5

Bellona x (136 Cantata x Mad. Lef.)	S-20186-2	2	Darwin Hybrid			40		
Pax x (137 Cantata x Mad. Lef.)	S-20253-1	5	Darwin Hybrid	10	50	30	15	30

Appendix II: Flow cytometry result by crossing between diploid mothers and selected diploid fathers which have 2n pollen. There were 2 seedlings have been tested in each cross.

Patental polyploidy	Mother	Father	Ploidy 2n	4n	3n	6n	Percent % C2	% C4	% C3	% C6
G2x G2x	080054 Escape	20170-7 Bellona	2x				100%			
G2x G2x	080049 Michael	20170-7 Bellona	2x				100%			
G2x G2x	080062 Ile de France	20170-7 Bellona	2x				100%			
G2x G2x	080062 Ile de France	20170-7 Bellona	2x				100%			
G2x G2x	080062 Ile de France	20170-7 Bellona	2x				100%			
G2x G2x	080062 Ile de France	20170-7 Bellona	2x				100%			
G2x G2x	070016 Bolroy Silver	20170-7 Bellona			3x				100%	
G2x G2x	080064 Leen v/d Mark	S-20186-2 Bellona			3x				100%	
G2x G2x	080064 Leen v/d Mark	S-20186-2 Bellona			3x				100%	
G2x G2x	080064 Leen v/d Mark	S-20186-2 Bellona			3x				100%	
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				28%			
G2x G2x	080063 White Marvel	S-20186-2 Bellona	2x				100%			
G2x G2x	080051 Royal Virgin	20241-2 Pax	2x				100%			
G2x G2x	080051 Royal Virgin	20241-2 Pax	2x				100%			
G2x G2x	080051 Royal Virgin	20241-2 Pax	2x				100%			
G2x G2x	080051 Royal Virgin	20241-2 Pax	2x				100%			
G2x G2x	080052 Ayake	20241-2 Pax	2x				100%			
G2x G2x	080052 Ayake	20241-2 Pax	2x				100%			
G2x G2x	080052 Ayake	20241-2 Pax	2x				100%			
G2x G2x	080052 Ayake	20241-2 Pax	2x				100%			

G2x G2x	080052 Ayake	20241-2 Pax	2x				100%			
G2x G2x	080062 Ile de France	20241-2 Pax	2x				100%			
G2x G2x	080062 Ile de France	20241-2 Pax	2x				100%			
G2x G2x	080062 Ile de France	20241-2 Pax	2x				100%			
G2x G2x	080062 Ile de France	20241-2 Pax	2x				100%			
G2x G2x	080064 Leen v/d Mark	20241-2 Pax	2x				100%			
G2x G2x	080064 Leen v/d Mark	20241-2 Pax	2x				100%			
G2x G2x	080054 Escape	20241-2 Pax	2x				100%			
G2x G2x	080063 White Marvel	20168-3 Bellona	2x				67%		33%	
G2x G2x	080063 White Marvel	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	070013 AC 12	20168-3 Bellona	2x				100%			
G2x G2x	080063 White Marvel	20190-4 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20190-4 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20190-4 Bellona	2x				100%			
G2x G2x	080064 Leen v/d Mark	20190-4 Bellona	2x				100%			
G2x G2x	080054 Escape	20230-9 Ile de France	2x				100%			
G2x G2x	080054 Escape	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	060057 Arcate	20230-9 Ile de France	2x				100%			
G2x G2x	?	?	2x				100%			
G2x G2x	?	?	2x				100%			

G2x G2x	?	?	2x				100%			
G2x G2x	?	?	2x				100%			
G2x G2x	?	?	2x				100%			
G2x G2x	G2x	GF 20180-3 2n pollen	2x		3x		63%		37%	
G2x G2x	G2x	GF 20180-3 2n pollen	2x				100%			
G2x G2x	G2x	GF 20180-3 2n pollen	2x		3x		64%		36%	
G2x G2x	G2x	GF 20180-3 2n pollen	2x				100%			

* 080054 Escape; 080049 Michail; 080062 Ile de France; 070016 Bolroy Silver; 080064 L v/d Mark; 080063 White Marvel; 080055 Royal Virgin; 080052 Aayke; 070013 AC 12; 060057 Arcate; 0912869 control; 20170-7 Bellona x (112 Juan x Cantata); S-20186-2 Bellona x (136 Cantata x Mad. Lef.); 20241-2 Pax x (102 Juan x Cantata); 20168-3 Bellona x (110 Juan x Cantata);20190-4 Bellona x (143 Princeps x Mad. Lef.); 20230-9 Ile de France x (155 Princept x Cantata)

Appendix III:Flow cytometry result by crossing between triploid mothers and selected diploid fathers which have 2n pollen. There were 2 seedlings of each crossing have been tested.

Patental	Mother	Father	Ploidy				Percent				Remark	
polyploidy			2n	4n	3n	6n	% C2	% C4	% C3	% C6		
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			18	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			18	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			19	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x		5x		46%		54%	19	25
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			18	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x		5x		67%		33%	18	26
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			18	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x		5x		68%		32%	18	25
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen				5x				100%	26	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen				5x				100%	25	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			18	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x		5x		30%		70%	19	25
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			19	
G3x G2x	070016 Bolroy Silver	S-20253-1 Pax 2n pollen		4x				100%			18	
G3x G2x	080049 Michail	S-20253-1 Pax 2n pollen	2x		3x		59%		41%		11	15
G3x G2x	080049 Michail	S-20253-1 Pax 2n pollen	2x				100%				10	
G3x G2x	080049 Michail	S-20253-1 Pax 2n pollen	2x				100%				11	
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x				100%			18	
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x		5x		46%			19	24
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x		5x			58%	42%	18	25
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x		5x		26%			19	24
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x					100%		18	
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x					100%		17	
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x		5x			54%	46%	17	25
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x		5x		65%	35%		17	24
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x					100%		17	
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x					100%		18	
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x					100%		18	
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x		5x		75%			18	23
G3x GF2x	BolroySilver	20180-3 2n pollen		3-4x		5x		19%	81%		18	24
G3x GF2x	BolroySilver	20171-8 2n pollen				5x		100%			24	
G3x GF2x	BolroySilver	20171-8 2n pollen		3-4x					100%		17	
G3x GF2x	BolroySilver	20171-8 2n pollen				5x				100%	24	
G3x GF2x	BolroySilver	20171-8 2n pollen		3-4x				100%			18	
G3x GF2x	BolroySilver	20171-8 2n pollen		3-4x				100%			18	
G3x GF2x	BolroySilver	20171-8 2n pollen				5x		100%			24	
G3x GF2x	BolroySilver	20171-8 2n pollen		3-4x		5x			44%	56%	17	25
G3x GF2x	BolroySilver	20171-8 2n pollen		3-4x		5x		72%		28%	18	24

G3x GF2x	BolroySilver	20171-8 2n pollen		3-4x					100%		17	
G3x GF2x	BolroySilver	20171-8 2n pollen		3-4x					100%		18	

Appendix IV: Original flow cytometry testing result.

Genotype	Number	Treatment	Ploidy 2n	4n	8n	Percent % C2	% C4	% C8
TT	062433	None	2x			100%		
TT	062433	0.001%	2x			100%		
TT	062433	0.001%	2x			68%	32%	
TT	062433	0.001%	2x			100%		
TT	062433	0.003%		**4x**			100%	
TT	062433	0.003%		**4x**			95%	5%
TT	062433	0.003%	2x			100%		
OT	082154	0.001%	2x			100%		
OT	082154	0.001%		**4x**			66%	34%
OT	082154	0.001%		**4x**			52%	48%
OT	082154	0.003%	2x			36%	64%	
OT	082154	0.003%	2x			27%	73%	
TT	061098	0.003%	2x			78%	22%	
TT	061098	0.003%	2x			100%		
TT	061098	0.003%	2x			80%	20%	
TT	061098	0.003%	2x			100%		
TT	061098	0.001%	2x			100%		
TT	061098	0.001%	2x			100%		
TT	061098	0.001%		**4x**			91%	9%
TT	061098	0.001%	2x			54%	46%	
TT	061098	0.001%	2x			97%	3%	
TT	061097	0.001%	2x			95%	5%	
TT	061097	0.001%	2x			100%		
TT	061097	0.001%	2x			100%		
TT	061097	0.001%	2x			100%		
TT	061097	0.001%		**4x**			100%	
TT	061097	0.001%	2x			100%		
TT	061097	0.003%		**4x**			100%	
TT	061099	0.003%	2x			100%		
TT	061099	0.003%		**4x**			45%	55%
TT	061099	0.003%			**8x**			100%
TT	061099	0.003%	2x			100%		
TO	082171	0.003%	2x			91%	9%	
TO	082171	0.001%	2x			92%	8%	
			25	8	1		**Total**	34
			74%	24%	3%			100%